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TOBACCO STUNT: INVESTIGATIONS ON THE COMPLEX OF DISEASE AGENT, FUNGUS VECTOR AND PLANT HOST.

by

PETER G. ALDERSON

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Tobacco Stunt: Investigations on the Complex of Disease Agent, Fungus Vector and Plant Host submitted by Peter G. Alderson in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Pathology.

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Date 20th October 1975

"When I use a word, Humpty Dumpty said,.....
it means just what I choose it to mean - neither more
nor less."

(Through the Looking Glass, Lewis Carroll)

ABSTRACT

The 3 biological entities involved in tobacco stunt disease were studied individually and together to determine their inter-relationships. Tobacco stunt agent (TSA) was acquired by *Olpidium brassicae* zoospores *in vivo* but not *in vitro*. At least 200 zoospores per ml were required for TSA transmission. Tobacco plants older than 5 weeks were susceptible to *O. brassicae* but apparently resistant to disease development.

Temporary remission of disease symptoms occurred in plants incubated at 25°C, whereas at 33°C they were freed of stunt. Infected plants grown at 17°C developed severe stunt symptoms, and the highest TSA infectivity, bioassayed on *Chenopodium amaranticolor*, was recovered from plants showing early systemic symptoms. Temperature affected the number of lesions produced on *C. amaranticolor*, the optimal temperature for lesion number was between 17 and 21°C. At 33°C no lesions developed. Treatment of infected tobacco plants at different stages of symptom development with antibiotics did not produce remission of symptoms.

TSA in tobacco sap was extremely unstable. Longevity was 2 hours at 20°C, and 24 hours at 4°C. Dilution end point was 1:100 and thermal inactivation occurred at 35°C for 10 minutes. TSA infectivity was low in sap extracted in buffers of low pH or of high molarity. TSA infectivity in sap was reduced by addition of bentonite, Mg-bentonite, protease and RNase. Yeast RNA partially stabilised TSA infectivity, and DNase had no effect on TSA. Attempts to isolate infectious TSA-RNA were unsuccessful.

Particles 15 to 50 nm in diameter were observed in non-infectious preparations following cycles of PEG 6000 precipitation. Infectivity was retained only after one treatment of the sap with PEG. Attempts to

visualise TSA in cells of *Olpidium* and of tobacco were unsuccessful.

No differences were observed between the life-cycles, morphology and ultrastructure of *O. brassicae* with and without TSA. Zoospores attached to tobacco roots retracted the flagellum by a 'wrap-around' method. Callose materials were deposited between the host cell wall and plasmamembrane at the site of penetration by the fungal cytoplasm. During the first 24 hours after penetration the thallus was separated from the host cytoplasm by only a single membrane. Scanning EM was used to examine stages in the life-cycle of *O. brassicae*. Resting sporangia possessed a thick, undulating, multilayered wall which had 5 and 6-sided facets. Lipid bodies were numerous at the periphery of the cytoplasm of resting sporangia and formed the sculptured outline observed after freeze-fracturing.

Internal and external symptoms of tobacco stunt were associated with TSA infection alone. *Olpidium brassicae* was non-pathogenic in tobacco, and no microorganisms were observed in the vascular tissues of stunted tobacco. Phloem tissues exhibited hyperplasia. Xylem cells had thin walls, were irregularly arranged and contained some gum-like material. Chloroplasts in leaf cells were distorted by large starch granules, and there was a higher incidence of lomasome-like bodies in stunted tobacco than in healthy plants.

Results from this study suggest that infectious TSA contains RNA which may possess a protein coat or be associated with host material. Evidence suggesting that TSA is distinct from tobacco necrosis virus, yellows disease agents and viroids is discussed.

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CHAPTER I

INTRODUCTION

During the last 2 decades considerable interest has been centred on the different types of associations between fungi and viruses. One type of association involves the fungus as the vector of virus and virus-like agents causing plant diseases. The possibility of fungi as vectors was suggested over the past 50 years, when attempts were made to inoculate plants with fungus spores or cultures originating from virus-infected plants (Bawden and Kassanis, 1947; Johnson and Jones, 1943; McKinney *et al.*, 1925; Nelson, 1932). However, actual demonstration of vector relationships was not achieved until after 1960, when the chytrid fungus *Olpidium brassicae* (Wor.) Dang. was shown to be the vector of the virus and virus-like agents which cause tobacco necrosis (Teakle, 1962), tobacco stunt (Hiruki, 1965), and lettuce big-vein (Campbell and Grogan, 1963; Campbell *et al.*, 1961; Tomlinson and Garrett, 1962).

The fungus transmission of soil-borne virus and virus-like agents is an area of active research because the diseases caused by these agents are economically important. Crops affected include cucumber, melon, lettuce, potato, tobacco and cereals. Plant viruses are biologically unique in having fungi as vectors, and these vectors have only been demonstrated in Oomycete fungi. Successful transmission by these vectors depends upon specific relationships in the complex of host plant, disease agent and fungus vector.

The 3 known disease agents transmitted by *O. brassicae* may be sub-divided on the basis of whether they are carried internally or externally by the fungus. Based on this criterion, a concept of persistent and non-persistent disease agent-vector relationships has been proposed (Campbell and Fry, 1966). *Olpidium brassicae* is the only fungus known to be involved in both persistent and non-persistent agent-vector associations, and therefore presents an ideal model system for studying fungus transmission.

Stunt disease of tobacco is caused by an infectious agent which has defied all attempts at its characterisation since the disease was first recorded in 1943 (Hiruki, 1965). Reports of virus particles associated with stunt disease (Hidaka, 1954; Hidaka *et al.*, 1956) have not been confirmed. In the literature this disease agent is referred to as tobacco stunt virus. The virus etiology of this disease has been based on (i) the development of virus-like symptoms in infected tobacco and transmission of the disease agent by grafting (Hidaka *et al.*, 1956), (ii) transmission by sap from diseased plants (Hiruki, 1964), and (iii) the production of antiserum from partially purified preparations of stunt agent (Hiruki, 1975). Since the infectious agent of tobacco stunt disease has not been isolated and visualised, it is still premature to call it a virus. The cells of host plant and vector have not been examined for other incitants of plant diseases e.g. viroids, mycoplasmas, rickettsias and bacteria, which can cause plant diseases with virus-like symptoms. Therefore, in this study, the causal agent of tobacco stunt disease is referred to as 'tobacco stunt agent'.

The persistent association of tobacco stunt agent with its vector suggests that it is carried internally by different stages of

the fungus. Only *in vivo* acquisition of stunt agent by *O. brassicae* has been demonstrated (Hiruki, 1965). Resting sporangia of the fungus retain infectious stunt agent for at least 12 years, and the results of chemical and physical treatments applied to these resting sporangia indicated that they are capable of transmitting tobacco stunt agent as long as the fungus remains viable (Hiruki, 1972). In contrast to this stable association of disease agent and vector, tobacco stunt agent appears to be extremely unstable in the tobacco host, and in sap extracts (Hidaka and Hiruki, 1958; Hiruki, 1975).

The non-persistent association of tobacco necrosis virus with *O. brassicae* has been studied extensively (Kassanis and MacFarlane, 1965; MacFarlane, 1968; Temmink, 1971; Temmink *et al.*, 1970). In contrast, comparatively little is known about the persistent relationships of tobacco stunt and lettuce big-vein disease agents with *O. brassicae*. The sap transmission of tobacco stunt agent confers certain advantages in the use of this disease agent, rather than lettuce big-vein agent, for studying a persistent disease agent-fungus vector relationship.

The objectives of this study are as follows:

1. To demonstrate the transmission of tobacco stunt agent by zoospores of *O. brassicae*.
2. To characterise tobacco stunt agent.
3. To investigate the morphology and ultrastructure of stages in the life-cycle of *O. brassicae*, relevant to its role as vector of tobacco stunt agent.
4. To investigate the histological and cytological aberrations following infection of tobacco with *O. brassicae* and tobacco stunt agent.

In the present study, investigations have been made on the complex of 3 biological entities involved in stunt disease of tobacco. This study has been maintained on a broad basis, because a knowledge of the inter-relationships between entities as well as a knowledge of each entity is required in understanding the complex nature of this particular system. A re-examination of stunt disease agent was included because it has not been convincingly demonstrated to be a virus. Attempts were made to visualise stunt agent in the fungus vector and in host plant tissues. A study of the aberrations in tissues and cells of stunt infected tobacco was included to determine the effects of stunt agent and *Olpidium* infection separately, and to locate the tissues where stunt agent might be present.

CHAPTER II

LITERATURE REVIEW

A. SOIL TRANSMISSION OF PLANT VIRUSES

The concepts that plant viruses can survive in soils solely by adsorption to colloidal particles (Miyamoto, 1959), and that infection of plant roots is achieved by semi-mechanical means, have largely been outdated by the demonstration of associations between soil-borne viruses and biological agents present in the soil. Such associations readily explain the survival and persistence of some virus and virus-like disease agents in soil.

Mayer in 1886 originally proposed the idea that plant viruses may be soil-borne. He suggested that the causal agent of tobacco mosaic should be looked for in the soil. Beijerinck (1898) tested this theory by growing seedlings in soil which previously contained infected plants. These seedlings contracted the disease. The subject of soil-borne viruses received little attention until 20 years ago, when the modes of transmission of some serious virus diseases could not be determined. In 1958, Hewitt *et al*. published the first report of an ectoparasitic nematode as the vector of a soil-borne plant virus. This was soon followed by claims for vector-like associations between the fungus, *Olpidium brassicae*, and certain virus and virus-like disease

agents (Campbell *et al.*, 1961; Hidaka, 1960; Teakle, 1960; Tomlinson *et al.*, 1962).

Cadman (1963a, 1963b) classified soil-transmitted viruses into 2 groups on the basis of whether they survived air-drying of the soil. Those surviving the treatment were considered fungus transmitted, whereas most of those losing transmissibility were considered nematode transmitted. However, this classification was not realistic since tobacco necrosis virus was fungus transmitted but lost its transmissibility in air-dried soil. Identity of the vector and the vector-virus relationship were considered to be more suitable for classification of soil-borne viruses (Grogan and Campbell, 1966). The importance of soil transmission of plant viruses during the 1960's was reflected by the number of review articles written (Cadman, 1963a, 1963b; Harrison, 1960, 1967). This subject has now expanded to the extent that specific areas, e.g. fungus transmission, have been reviewed separately (Grogan and Campbell, 1966; Teakle, 1967, 1969, 1972).

B. FUNGUS TRANSMISSION OF PLANT VIRUSES AND VIRUS-LIKE AGENTS

The first suggestion that soil fungi may act as vectors of viruses was made by McKinney (1930), who was working on transmission of soil-borne wheat mosaic. Many unsuccessful attempts to transmit plant viruses by fungi were made during the following years. At least 18 fungi were tested as vectors using different inoculation methods (Teakle, 1969), e.g. inoculation of plants with fungus spores or cultures from virus-infected plants; simultaneous application of

virus and fungus spore suspensions to leaves; and growing healthy and virus-infected plants together in soil inoculated with a fungus. In 1958, an association between *O. brassicae* and big-vein disease of lettuce was demonstrated (Fry, 1958; Grogan *et al.*, 1958), but the fungus alone was considered to cause the disease. In the following years it was shown that lettuce big-vein was caused by a graft-transmissible agent, and the association with *Olpidium* was claimed to be a virus-vector relationship (Campbell, 1962; Campbell *et al.*, 1961, Campbell and Grogan, 1963; Tomlinson and Garrett, 1962). *Olpidium brassicae* was also demonstrated to be the vector of tobacco necrosis virus (Teakle, 1962) and tobacco stunt agent (Hiruki, 1965). Prior to these reports, claims of possible virus-vector relationships between *O. brassicae* and the agents of tobacco necrosis and tobacco stunt had already been made (Hidaka, 1960; Hidaka and Tagawa, 1962; Teakle, 1960).

To date, associations have been reported between 6 fungi and 9 plant disease agents, 6 of which are known to be viruses (Table 1). As a group, fungus transmitted viruses are heterogeneous, both in morphology and in properties (Teakle, 1972). The fungus vectors are all Oomycetes, and possess similar life-cycles. They all produce motile zoospores which are the potential virus or disease agent vectors. These zoospores are released from thin-walled sporangia and from thick-walled resting sporangia. *Pythium ultimum* is, however, an exception since zoospores are only produced from sporangia developed from oospores. The thick walled resting sporangia (and oospores) of these fungi explain the persistence of these vectors and associated disease agents in soil. Except for potato

TABLE 1. Vector relationships of fungi and virus or virus-like disease agents

Fungus	Disease caused by agent	Identity & size (nm) of agent	Location of agent in vector
Olpidium brassicae	Tobacco necrosis	Polyhedral virus; 26-30	External
O. brassicae	(Tobacco necrosis - satellite virus)	Polyhedral virus; 17	External
O. brassicae	Tobacco stunt	?	Internal
O. brassicae	Lettuce big-vein	?	Internal
O. cucurbitacearum	Cucumber necrosis	Polyhedral virus; 30	External
Polymyxa graminis	Soil-borne wheat mosaic	Rigid rod virus; 160 x 25	Internal
Synchytrium endobioticum	Potato X	Flexuous rod virus; 515 x 13	Internal
Spongospora subterranea	Potato mop top	Rod virus 300 & 150 x 20	Internal
Pythium ultimum	Pea false leaf roll	?	?

virus X and pea false leaf roll agent, the remaining agents listed in Table 1 depend upon their fungus vector for spread. Thus the vectors are usually present with the diseased plants.

The different vector relationships have been divided into the disease agents which are carried internally and those carried externally (Campbell and Fry, 1966). This grouping indicated differences in the mode of agent acquisition by the vector, and in the survival of the agent in soil. The possibility of multiplication in the vector was also considered. *Olpidium brassicae* was the only known vector which illustrated both types of disease agent associations. An internal or persistent association was shown by lettuce big-vein agent. The agent was acquired *in vivo* by *O. brassicae* during one life-cycle of the fungus in big-vein infected lettuce roots (Campbell and Grogan, 1964). The resting sporangia of *O. brassicae*, which carried big-vein agent, survived strong acid and base treatments and still continued to transmit the infectious agent (Campbell, 1962; Campbell and Fry, 1966). Likewise, the infectious agent of tobacco stunt was also shown to have an internal and persistent association with *O. brassicae* (Hiruki, 1965, 1968, 1972).

Tobacco necrosis virus is an example of a disease agent which has an external or non-persistent association with its fungus vector. Zoospores of *O. brassicae* acquire TNV *in vitro*, and the virus enters the host root at the same time as the vector (Teakle, 1962; Kassanis and MacFarlane, 1964; Campbell and Fry, 1966). Zoospores previously exposed to virus were prevented from transmitting TNV by treatment with concentrated antiserum (Kassanis and MacFarlane, 1964). This result suggested that TNV was surface-borne, and the firmness of the association was emphasised. More recently, Temmink *et al*. (1970), using the electron microscope, observed virus particles attached to

washed zoospores after incubating in a TNV preparation. This strengthened the hypothesis that TNV was carried externally by *O. brassicae* zoospores. The non-persistent association of TNV and its vector was demonstrated by the treatment of resting sporangia from virus-infected roots with strong acid, which resulted in loss of ability to transmit the virus (Campbell and Fry, 1966).

Another species of *Olpidium*, *O. cucurbitacearum*, has been shown to be a virus vector (Dias, 1970a, 1970b). Cucumber necrosis virus was acquired *in vitro*, but was not carried internally by resting sporangia. This virus-vector relationship was suggested to be similar to that of TNV and *O. brassicae*.

Polymyxa graminis (Led.) has been shown to transmit soil-borne wheat mosaic virus (WMV) (Brakke *et al.*, 1965; Estes and Brakke, 1966). Treatment of zoospores with antiserum, and resting sporangia with strong acid or strong base did not remove the ability of the fungus to transmit the virus. An internal and persistent virus-vector relationship for WMV - *P. graminis* was suggested (Rao, 1968; Rao and Brakke, 1969).

Transmission of potato virus X (PVX) by *Synchytrium endobioticum* (Schilb) Perc. from potato tubers to healthy tubers was reported under glasshouse conditions (Nienhaus and Stille, 1965). Acquisition was *in vivo*, and the virus was suggested to be carried internally by the zoospores.

Potato mop top virus (PMTV) has been shown to be transmitted by resting sporangia of *Spongospora subterranea* (Wallr.) Lagerh. (Calvert and Harrison, 1966; Jones and Harrison, 1969). The virus was suggested to be carried internally, because viruliferous spore

balls retained their ability to transmit PMTV after air-drying. Also, infested soils always contained resting sporangia of the fungus.

Pea false leaf roll has been suggested to be fungus transmitted based on the following evidence: (i) healthy seedlings became infected in infested soil, (ii) infection was prevented by soil sterilisation or treatment with fungicides, and (iii) pure cultures of *Pythium ultimum* Trow from diseased plant roots induced the disease in healthy plants (Thottappilly and Schmutterer, 1968). However, the evidence presented was only preliminary, since the possibility that the fungus may have induced the disease was not excluded. This disease agent was also considered to be transmitted mechanically, by seed, and by aphids.

During the last 15 years a large volume of literature on the subject of fungus transmission of plant viruses and virus-like disease agents has been published. However, it is probable that still more disease agent - fungus vector associations will be demonstrated in the future. Oat mosaic virus has been associated with both *P. graminis* and *O. brassicae*, and the soil-borne transmission of tomato bushy stunt virus was inhibited by fungicide (Lovisolo, 1966). Also a soil-borne virus, possibly involving a fungus vector, was reported for wheat spindle streak mosaic (Slykhuis, 1970). The agent of freesia leaf necrosis was also reported to be soil-borne (Van Dorst, 1975).

Olpidium brassicae has been used extensively for investigations on virus-vector relationships because it transmits disease agents of persistent and non-persistent associations. The factors involved in the specificity of transmission of disease agents by *O. brassicae*

have been investigated. Teakle and Hiruki (1964) determined that host specificity for multiplication of *O. brassicae* did not determine the specificity of the vector to transmit TNV. It was suggested that non-vector strains of zoospores were inefficient in acquiring TNV, since less virus infectivity was recovered from non-vector strains than from vector strains. This result was confirmed by electron microscopic observation of more particles of virus attached to zoospores of vector strains than to non-vector strains (Temmink, 1971; Temmink *et al.*, 1970). Introducing the host plant into the relationship of disease agent and vector means that further inter-relationships have to be considered for successful fungus transmission of the disease agent (Temmink, 1971). The host specificities of *O. brassicae* with tobacco stunt agent (Hiruki, 1967), and with tobacco necrosis virus (Kassanis and MacFarlane, 1965) have been reported. Tobacco stunt and tobacco necrosis both possess wide host ranges. In contrast lettuce big-vein is believed to have a narrow host range, which may in part be due to the lack of a suitable technique for sap transmission.

C. *OLPIDIUM BRASSICAE*

The first description of the chytrid fungus, *O. brassicae*, was made by Woronin (1878), and Dangeard (1886) assigned the fungus to the genus *Olpidium*. On the basis of host specialisation and transmission of the lettuce big-vein agent, it was proposed that the cabbage and lettuce isolates of *O. brassicae* be reclassified as *Pleotrachelus brassicae* and *P. virulentus* respectively (Sahtiyanci, 1962; Sahtiyanci

et al., 1960). However, it is now generally accepted that the fungus belongs to the genus *Olpidium*, and that the different isolates belong to the single species, *O. brassicae* (Hiruki, 1965; MacFarlane, 1968; Teakle and Hiruki, 1964; Temmink, 1971).

The morphology and life-cycle of *O. brassicae* have been investigated extensively with the light microscope (Bensaude, 1923; Kole, 1954; Jacobsen, 1943 - cited by Temmink, 1971; Sahtiyanci, 1962; Sahtiyanci *et al.*, 1960; Sampson, 1939; Van der Meer, 1926). Garrett and Tomlinson (1967) examined 8 isolates of *O. brassicae* from lettuce and cabbage roots, and concluded that, although zoospore diameter, flagellum length, and pathogenicity were relatively constant for each isolate, these isolates studied should still be considered as the same species. The fungus has three stages in its life-cycle (Plate 4) i.e. zoospore, zoosporangium and resting sporangium. Zoospores are motile and possess a round or pear-shaped body, 3 to 4 µm in diameter, and a single posterior flagellum 13 to 21 µm in length. The whiplash on the flagellum is 2 µm long (Teakle, 1967). The variations in dimensions reported were due to the different lettuce and cabbage isolates examined. Zoospores of cabbage isolates were reported with the body 3 µm in diameter and the flagellum 16 to 17 µm in length (Garrett and Tomlinson, 1967; Sampson, 1939), whereas those of lettuce isolates had a body 3 to 4 µm in diameter, and flagellum up to 21 µm in length.

Zoosporangia and resting sporangia develop in the epidermal and cortical cells of plant roots following the successful infection of the cells by zoospores. Zoosporangia vary in shape from spherical,

9-20 µm in diameter, to elongate, 11 to 220 µm in length and 11 to 45 µm in width. Zoosporangia mature in 3 to 4 days, and zoospores are released through exit tubes. Sometimes there are more than 1 exit tube per zoosporangium. Cabbage isolates of *O. brassicae* possess long exit tubes, 5 to 70 µm in length, whereas those of lettuce isolates are only 3 to 4 µm in length (Teakle, 1967).

The shape of resting sporangia varies from round, 9 to 25 µm in diameter, to ovoid, 17 to 64 µm in length and 13 to 19 µm in width. During the first 2 days after penetration of the host cell by a zoospore, the developing resting sporangium resembles a zoosporangium. However, after 3 days the characteristic thick undulating wall of a resting sporangium is visible. This stage of the fungus is able to survive desiccation and other unfavourable environmental conditions. Zoospore release from resting sporangia occurs via exit tubes after a minimum maturation period of 16 days (Teakle, 1967). Some investigators have proposed that resting spore formation represents the sexual phase in the life-cycle of *O. brassicae*, as based on observations of zoospores with more than one flagellum, and on the fusion of motile zoospores (Kole, 1954; Sahtiyanci, 1962). Similar observations of 'double' zoospores have been made by other workers, however, no evidence was obtained for the subsequent development of resting sporangia from these zoospores (Garrett and Tomlinson, 1967; Temmink and Campbell, 1969a; Tomlinson and Garrett, 1964).

The ultrastructure of zoospores and zoosporangia of a lettuce isolate of *O. brassicae* has been studied in detail (Temmink and Campbell, 1968, 1969a, 1969b; Temmink, 1971). The stages of encystment and penetration of cabbage roots by a cabbage isolate of *O. brassicae*

zoospores have also been reported (Lesemann and Fuchs, 1970a, 1970b). The zoospore body was pyriform and surrounded by a single membrane which was continuous with the membrane around the flagellum. The typical 9+2 arrangement of axonemal fibrils within the flagellum was observed. The zoospore cytoplasm contained ribosomes, which were evenly distributed, and mitochondria located around the single nucleus. The axoneme lacked a terminal plate and was in contact with the kinetosome. A cross-banded structure, the rhizoplast, approximately 0.35 x 1 μm, was observed between the kinetosome and the nucleus.

After attachment to host plant roots, zoospores were observed encysted on the roots (Lesemann and Fuchs, 1970a; Temmink, 1971; Temmink and Campbell, 1969b). Temmink (1971) concluded that retraction of the flagellum occurred before the zoospore body was firmly attached to the root surface, because such stages were not observed after processing and sectioning for electron microscopy. Flagellum retraction was suggested to occur by a reeling-in method (Temmink, 1971; Temmink and Campbell, 1969b). The accumulation of membranous material inside the encysted zoospore cytoplasm was thought to have originated from the flagellum. The location of the rhizoplast inside encysted zoospores was also used as evidence for the reeling-in of the flagellum. In contrast to this report, Lesemann and Fuchs (1970a) suggested that retraction occurred by a wrap-around method. Previously Koch (1968) suggested, from light microscope observations, that retraction occurred by either the reeling-in or the wrap-around method.

During encystment of the zoospore body on the host root a cyst wall was deposited exterior to the body membrane. The axonemal fibrils

and rhizoplast, initially observed inside the body cytoplasm, disintegrated (Lesemann and Fuchs, 1970a). Vesicles produced by a dictyosome aggregated at the site of attachment of the cyst to the host cell wall. After 2 hours of encystment, a large vacuole developed in the cyst, distal to the site of attachment. This vacuole was suggested to be involved in the movement of cyst cytoplasm into the host cell after penetration of the host cell wall (Temmink, 1971; Temmink and Campbell, 1969b). Prior to penetration, a thickening of the host cell wall was reported. Material was deposited between the host plasmamembrane and host cell wall adjacent to the attached cyst. This deposition of material was termed a 'papillum' (Temmink and Campbell, 1969b) and a 'callosity' (Lesemann and Fuchs, 1970b).

Penetration of the thickened host cell wall and plasmamembrane occurred after 2 hours of encystment. The cyst cytoplasm was then located in the host cell and separated from the host cytoplasm by only a single membrane (Lesemann and Fuchs, 1970b; Temmink and Campbell, 1969b). During the following 24 hours the fungal thallus was limited by only this single membrane. At 36 hours a thallus wall was deposited exterior to this membrane (Temmink and Campbell, 1968). The thallus cytoplasm contained mitochondria, ribosomes and lipid bodies, and was multinucleate. Multivesicular bodies were also present, and thought to be involved in the cleavage of the cytoplasm into zoospore protoplasts. After 72 hours mature thalli contained zoospores which were fully differentiated. Temmink and Campbell (1968) suggested that thalli developing into resting sporangia did not become multinucleate between 24 and 48 hours after infection. Instead, storage material was observed in these thalli.

Some reports consider *O. brassicae* to be a pathogen causing disorders such as damping-off of crucifers (Woronin, 1878), and chlorosis of spinach (Rich, 1959). Van der Meer (1926) showed that *Rhizoctonia* was probably the cause of damping-off of crucifers. In some light microscope studies the possible pathogenicity of *Olpidium* was considered (Britton and Rogers, 1963; Rich, 1959; Sahtiyanci, 1962; Wolf, 1935). The swollen tips of root hairs associated with *O. brassicae* infection was reported by Sahtiyanci *et al.* (1960). However, *O. brassicae* is considered by others to be a parasite rather than a pathogen (Grogan and Campbell, 1966). Roots of lettuce, crucifers and cereals were heavily infected with virus-free *O. brassicae*, and the only symptom observed was a slight reduction in the vegetative growth of the plants. Grogan and Campbell (1966) surmised that a fungus with a host-parasite relationship would more likely be a virus vector than a fungus which was highly pathogenic.

D. TOBACCO STUNT

A severe disease occurring in tobacco seedbeds was first described by Nakamura and Tsumagari in 1943 and referred to as 'tobacco shrink' (Hiruki, 1965). This disease, now known as tobacco stunt, has only so far been reported in Japan. The soil-borne nature of the disease agent (TSA) was established (Hidaka *et al.*, 1956; Uozumi, 1954), and observations were made on the location of the agent in soil fractions, on symptomatology, on varietal differences in

disease resistance, on practical control methods, on the relation of temperature to symptom expression, and on the heat therapy of diseased plants (Hidaka and Hiruki, 1958; Hidaka *et al.*, 1956; Uozumi, 1954). Tobacco stunt infectivity was not lost by air-drying infested soil or by treating infested soil with oxygen and carbon dioxide for 40 days. From these results it was suggested that TSA did not exist free or adsorbed to particles in soil, but was present in the body of a resistant vector. This vector was considered to be a fungus, since infested soil lost its infectivity after treatment with fungicides, but not after treatment with insecticides. A claim of the vector relationship of *O. brassicae* with TSA was made on the basis of the consistent association of the fungus in the roots of stunt-infected plants (Hidaka, 1960; Hidaka and Tagawa, 1962). Actual demonstration of the transmission of TSA by *O. brassicae* was reported by Hiruki (1965).

Attempts have been made to isolate and characterise the infectious agent of stunt disease. Hidaka (1954) isolated virus-like particles, 25 nm in diameter, but the infectivity obtained was not associated with stunt disease. Later, non-infectious virus-like particles, 18 nm in diameter, were reported in purified preparations obtained from tobacco tissues (Hidaka *et al.*, 1956). The disease agent was considered to be a virus on the evidence of disease symptoms, graft transmission of the infectious agent, and the isolation of virus-like particles. However, the presence of conventional virus particles, in the fungus vector or in the infected host, has not been convincingly demonstrated. Virus-like particles, 18 nm in diameter, were reported in the cytoplasm of cells in a pellet of 'viruliferous'

O. brassicae zoospores (Soejima and Hidaka, 1969). These particles were claimed to be tobacco stunt 'virus', however the identity of these particles was not established.

Successful mechanical transmission of TSA was reported by Hiruki (1964). The addition of certain chelating agents, in particular 1-phenylthiosemicarbazide, to the phosphate buffer during sap extraction was effective in stabilising TSA infectivity. A local-lesion assay host, *Chenopodium amaranticolor* Coste & Reyn., was also reported. Leaves of French bean, *Phaseolus vulgaris* L. var. Pinto, were used as a differential indicator host to detect possible contamination with tobacco necrosis virus. The development of mechanical transmission of TSA made it possible to investigate further the vector relationship between *O. brassicae* and TSA.

The acquisition of TSA by zoospores was shown to be *in vivo* (Hiruki, 1965). Zoospores, free of TSA, acquired TSA from the roots of tobacco previously infected by sap inoculation. Transmission of TSA by zoospores carrying the agent was prevented by heat and chemical treatments which killed the zoospores. Tobacco plants became infected with stunt when grown in stunt-infested soil which had been air-dried for 12 years, and when *O. brassicae* zoospores or resting sporangia were transferred from infected to healthy tobacco. However, direct application of TSA in sap to the roots of healthy tobacco did not result in infection. From all these results, it was suggested that TSA was carried inside *O. brassicae* zoospores. The persistent association of TSA with its vector was further demonstrated by the treatment of resting sporangia carrying TSA with heat, acid, base, UV irradiation, and drying. Infectivity of TSA was obtained from

treated resting sporangia as long as the fungus remained viable. This suggested a close and stable association between TSA and its vector, but multiplication in the vector may not occur because the fungus was freed of TSA by serial transfer on cowpea, *Vigna sinensis*, and no TSA was detected in roots inoculated with *Olpidium*/TSA (Hiruki, 1965, 1967).

Hiruki (1967) studied the host range of *O. brassicae* and TSA and concluded that host specificity to *O. brassicae*, as well as to TSA, influenced fungus transmission. Interrelationships between host, fungus vector and TSA were suggested to be complex. Zoospores of *O. brassicae* transmitted TSA to 35 plant species in 13 families. Plants were also tested for susceptibility to sap inoculation with TSA. Recently, the host range of mechanically transmitted TSA was extended to 41 species in 9 families (Hiruki, 1975). Some physical properties of TSA in sap extracts from infected tobacco were also reported. Evidence, based on the reaction of host plants, serological reaction and cross protection, was presented for the distinction between TSA and an isolate of TNV. Preliminary results of the effects of certain substances on TSA infectivity in tobacco sap indicated that TSA was extremely unstable and contained infectious ribonucleic acid (Hiruki *et al.*, 1974a).

E. BIOLOGICAL AGENTS CAUSING PLANT DISEASES WITH VIRUS-LIKE SYMPTOMS

During the last decade certain plant diseases with virus-like symptoms have been demonstrated to be caused by biological agents other than conventional viruses. These agents include viroids, Mollicutes-like organisms, rickettsias and bacteria. Since the nature of TSA is

still unknown, it is relevant to include a brief summary of some of the characteristics of these agents in this review of literature.

1. Viroids

The term viroid denotes a novel group of infectious agents which exist as free nucleic acids (Diener, 1971, 1972). This group of agents is characterised by the absence of conventional particles containing a protein component, and by genomes that are very small in contrast with those of plant viruses. Several plant diseases are known or have been suggested to be caused by such agents, i.e. potato spindle tuber (Diener and Raymer, 1967; Singh and Bagnall, 1968), chrysanthemum stunt (Diener and Lawson, 1973; Hollings and Stone, 1973), cadang-cadang disease of coconut palms (Randles, 1975), chrysanthemum chlorotic mottle disease (Romaine and Horst, 1975), citrus exocortis (Semancik and Weathers, 1972), and pale fruit of cucumber (Van Dorst and Peters, 1974). These diseases exhibit some virus-like symptoms but have not been associated with conventional virus particles. Lawson and Hearon (1971) attempted without success to localise virus in thin sections of chrysanthemums infected with stunt.

The viroid associated with potato spindle tuber has been investigated the most extensively. Certain characteristics appear to be common to most if not to all the known or suggested viroids causing plant diseases. Bioassay of these agents usually involves long incubation periods on systemic hosts. Transmission has been achieved by sap and by grafting. Treatment of crude sap or purified preparations of these agents with ribonuclease causes inactivation, whereas DNase has no effect. Likewise,treatments with organic solvents and phenol do not reduce

infectivity. Highly infectious preparations can be prepared by precipitation with ethanol following phenol extraction. High buffer molarity enhances infectivity of extracts containing these agents, and partially protects them against the action of RNase. Following high speed centrifugation, most infectious material remains in the supernatant, and fractionation by sucrose density gradient centrifugation occurs under conditions used for nucleic acids (Diener and Raymer, 1967). The majority of infectious potato spindle tuber viroid sediments at 10S indicating that it is an RNA of very low molecular weight (Diener and Raymer, 1969; Raymer and Diener, 1969). The heterogeneous sedimentation properties of this viroid are due to its association with host nuclei, especially the chromatin. Sedimentation heterogeneity was suggested for citrus exocortis (Semancik and Weathers, 1972), chrysanthemum stunt (Diener and Lawson, 1973) and chrysanthemum chlorotic mottle (Romaine and Horst, 1975). These agents are also thought to be associated with host materials, and are remarkably stable, low molecular weight RNA's.

2. Mollicutes-like organisms

One of the criteria used for identification of a mycoplasma is that it be cultured on artificial media. This requirement has not been met for suspected mycoplasmas which cause certain plant diseases. It was suggested that these agents should be referred to as Mollicutes-like organisms or yellows disease-associated agents until Koch's postulates have been fully met (Hayflick and Arai, 1973).

The first report of a suspected mycoplasma etiology for a plant disease was made by Doi *et al.* in 1967. Based on the observation of

microorganisms in the phloem of yellows diseased plants and the remission of symptoms by tetracycline antibiotics and not by penicillin, it was proposed that these diseases were caused by mycoplasma- or chlamydia-like organisms. Since then more than 50 diseases of the yellows type have been reported to be associated with these microorganisms (Davis and Whitcomb, 1971). A considerable amount of literature on this subject has accumulated over the last 8 years, including several review articles (Davis and Whitcomb, 1971; Hampton, 1972; Hull, 1971; Maramorosch *et al.*, 1970; Whitcomb and Davis, 1970).

Symptoms of diseases associated with Mollicutes-like organisms include vein-clearing of young leaves typical of virus diseases, virescence i.e. greening of petals, and phyllody i.e. conversion of petals to leaf-like structures. In some diseases proliferation of shoots occurs due to the loss of dormancy of axillary buds. Yellows diseases include aster yellows which, at one time thought to be caused by a virus, is one of the most widespread diseases of plants. It is not yet known whether the causal agent is identical in all the hosts reported, which include 300 species of plants in 48 families. Aster yellows is considered as a classic representative of yellows-type diseases in plants.

Yellows diseases are characterised by typical external disease symptoms, the presence of pleomorphic bodies in phloem cells (Hirumi and Maramorosch, 1973), transmission of the disease agents by grafting, and by leafhopper vectors, remission of symptoms by high temperatures and the remission of symptoms by tetracycline antibiotics (Asuyama and Iida, 1973; Ishiie *et al.*, 1967). The pleomorphic bodies observed in phloem cells include spherical bodies and filamentous forms ranging from 70 to 700 nm

in size. Some forms suggest division by binary fission or budding. A unit membrane, 7 to 12 nm thick consisting of electron-dense layers separated by an electron-lucent layer, surrounds each body. Ribosomes are present in the bodies, either scattered or clustered near the cell membrane, and strands of DNA are present in the central area.

The presence of Mollicutes-like organisms in phloem cells is accompanied by necrosis of the cells and abnormal deposition of callose materials (Dijkstra and Hiruki, 1974; Goszdziewski and Petzold, 1975; Hiruki and Dijkstra, 1973; Hiruki *et al.*, 1974b; Schneider, 1973). These aberrations of the phloem tissues can be detected by fluorescence microscopy and used to indicate the presence of mycoplasma-like agents in the tissues. Attempts to use negative staining of extracts from yellows diseased plants for the identification of presumptive mycoplasma agents have been unsuccessful, since it was not possible to distinguish the agents from artifacts associated with host materials (Wolanski, 1973).

The published reports showing an association between Mollicutes-like agents and yellows type diseases have ranged from electron microscope observations of these agents in infected plants to actual claims of mycoplasma isolations, growth on agar and the completion of Koch's postulates (Chen and Granados, 1970; Hampton *et al.*, 1969; Nayar and Ananthapadmanabha, 1970). However, these reports of cultivation of plant pathogenic mycoplasmas have since been shown to be due to contamination during culture. Hayflick and Arai (1973), in attempts to confirm these claims, were unable to culture mycoplasmas from aster yellows-diseased plants and leafhoppers.

A Mollicutes organism recently assigned to the Mycoplasmatales, has been successfully cultured from citrus plants with "stubborn"

disease (Abd El-Shafy *et al.*, 1972; Bové *et al.*, 1973). Based on helical morphology (Cole et al., 1973), rotary motility, bacteriophage infection, surface projections on the cytoplasmic membrane, gram-positivity and optimal temperature for growth close to 32°C, this microorganism has been assigned to the genus and species *Spiroplasma citri* in the order Mycoplasmatales. The agent of corn stunt disease is also known to be a type of spiroplasma (Davis and Worley, 1973).

Mollicutes-like organisms have also been associated with cultures of fungi (Heath and Unestam, 1974; Hendrix, 1974). It is not known whether these microorganisms are parasitic or symbiotic.

3. Bacteria and rickettsias

The possibility of rickettsias as agents of plant diseases was suggested by Davis and Whitcomb (1971). These agents, which are mainly parasitic on arthropods, comprise a group of small, pleomorphic, gram-negative bacteria. They are larger than mycoplasmas and chlamydiae, 0.5 to 0.8 by 1.0 to 2.0 μm, and thus retained by filters through which the others pass. Giannotti *et al.* (1970) reported rickettsias in dodder. Duval (1970) observed them in a myxomycete and found that treatment with chloramphenicol, but not with penicillin or tetracycline, freed the fungus of the microorganism.

The first plant disease suggested to be associated with a rickettsia-like organism was Pierce's disease of grapevines (Hopkins and Mollenhauer, 1973). This report was confirmed and a similar association of rickettsia with alfalfa plants affected with dwarf disease reported (Goheen *et al.*,1973). Previously Pierce's disease of grapevines was thought to be caused by a virus (Esau, 1948), and later

by a mycoplasma based on the suppression of disease symptoms by tetracyclines (Hopkins and Mortensen, 1971). However this disease is now considered to be caused by a rod-shaped gram-positive bacterium (Auger *et al.*, 1974), which has been isolated from infective leafhoppers, cultured on artificial media and which induces the symptoms of Pierce's disease when re-inoculated into healthy plants. This organism was restricted to the xylem of its host plant and to the tissues of its vector. So far it has not been possible to culture it from infected plant tissues.

The presence of rickettsia-like organisms has been reported for sugar cane ratoon stunt (Maramorosch *et al.*, 1973; Teakle *et al.*, 1973), for apple proliferation (Petzold *et al.*, 1973), for clover club leaf (Windsor and Black, 1973), and for phony peach disease (Goheen *et al.*, 1973; Nyland *et al.*, 1973). Microorganisms were detected in the vascular tissues of diseased plants and their identity was based on morphology, intracellular location, and absence in healthy plants. All these agents, except apple proliferation, have known leafhopper vectors, and disease symptoms include yellows, typical of mycoplasma infections, and dwarfing of plants. None of these agents have been successfully cultured from host tissues. The clover club leaf agent has been investigated more extensively than the other agents. Unlike the other agents, it was located in phloem cells and not in xylem, and was smaller in size. It possessed a double membrane, or a membrane plus a wall, and the remission of disease symptoms by penicillin suggested the presence of muramic acid in this wall. The organisms associated with phony peach disease resembled those occurring in grapevines infected with Pierce's disease, and may eventually be grouped

with this agent.

Esau (1948) reported a pattern of gum deposits and tyloses in the xylem of stems of grapevines inoculated with Pierce's disease agent. A similar host response was observed in the xylem of alfalfa infected with dwarf disease agent. Gum was also reported in xylem cells in peach tree roots affected with phony disease (French, 1974), and vascular discolourations were observed in ratoon stunted sugar cane below the apical region of shoots (Maramorosch *et al.*, 1973).

Chlamydiae have not yet been observed in plant tissues, but their presence in cicadellids suggests that they may eventually be located in plants (Davis and Whitcomb, 1971). These organisms are spherical, 200 to 1000 nm in diameter with a plasmamembrane and an outer wall containing muramic acid, similar to the wall of bacteria.

CHAPTER III

MATERIALS AND GENERAL METHODS

A. CULTURE OF *OLPIDIUM BRASSICAE* ISOLATES

The 2 isolates of *O. brassicae* (Wor.) Dang. used in this study were originally obtained from a single isolate from tobacco infected with tobacco stunt agent (hereafter referred to as TSA). One of the isolates (hereafter referred to as *Olpidium*) was freed of TSA by culturing on the roots of cowpea, *Vigna sinensis* (Hiruki, 1965). This isolate, and the isolate carrying TSA (hereafter referred to as *Olpidium*/TSA), had both been maintained as resting sporangia in dried powdered roots for 5 years.

The *Olpidium* and *Olpidium*/TSA isolates were cultured on roots of tobacco seedlings grown in sterilised quartz sand, inoculated with the above dried root powders containing resting sporangia, and watered with a half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Seedlings were either grown in microincubators (Hiruki, 1969) (Plate 1, B & C), or in clay pots inside plastic containers (Plate 1, A) when larger populations of the fungus were required. Plants were maintained in growth chambers at 17°C with 16 hours light period and 5,000 - 10,000 lux light intensity.

B. PREPARATION OF ZOOSPORE SUSPENSIONS

Light microscopy of *Olpidium* and *Olpidium*/TSA infected tobacco

roots permitted selection of roots containing abundant mature zoosporangia. Roots 2 to 3 weeks after inoculation with resting sporangia were usually suitable. These roots were washed free of sand in cold running water, and were then incubated in water at room temperature for 10 minutes to permit zoospore release. The resulting zoospore suspension was passed through Whatman No. 1 filter paper to remove any remaining sand particles and plant debris. The concentration of the suspension was estimated in units of zoospores/ml using a hemocytometer. When high concentrations of zoospores were required the suspension was centrifuged at 3,500g for 10 minutes (Sorvall RCB-2, SS 34 rotor) at 4°C, and the zoospore pellet resuspended in a small volume of distilled water.

C. CULTURE OF TOBACCO PLANTS

In this study, *Nicotiana tabacum* L. 'Bright Yellow' (hereafter referred to as tobacco) was used as the host plant because it is highly susceptible to both *Olpidium* and TSA. The fine, translucent roots of tobacco seedlings grown in sand culture permit light microscope observations on *Olpidium* in live roots.

Tobacco seed was germinated at 25°C on a 1:1 mixture of vermiculite and autoclaved 3:2:1 soil mix (3 parts loam: 2 parts peat: 1 part sand, hereafter referred to as soil mix). Seedlings approximately 2 weeks old were transplanted into sterilised plastic trays (ultraviolet irradiation for 2 hours) containing the same vermiculite / soil mix. Transplanted seedlings were then grown in a greenhouse maintained at 20° ± 2°C with 14 hours light period. During

the summer months the glass was coated with a shading compound to keep the light intensity below 15,000 lux. Two weeks after transplanting and at subsequent intervals of 2 weeks a high nitrogen, water soluble fertiliser (28-14-14, Plant Products Co. Ltd., Bramalea, Ontario) was applied to the plants.

D. INOCULATION OF TOBACCO SEEDLINGS WITH *OLPIDIUM* AND *OLPIDIUM*/TSA

A dip inoculation method (Hiruki, 1967) was used for the inoculation of tobacco roots with *Olpidium* zoospores. The roots of tobacco seedlings, approximately 2 weeks old, were washed free of soil and then incubated in zoospore suspensions containing at least 10^3 zoospores/ml. After one hour of incubation these roots were washed in water and the seedlings transplanted into vermiculite/soil mix in sterilised plastic trays.

E. INOCULATION OF TOBACCO WITH SAP CONTAINING TSA

Tobacco plants grown as outlined above were selected at the 2 or 3 leaves stage for inoculation with tobacco sap containing TSA. This sap was obtained by homogenising tobacco leaves, showing symptoms of stunt infection, with 0.01M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 0.001M 4-phenylthiosemicarbazide (4-PTC) (hereafter referred to as phosphate/PTC buffer) in a pre-cooled mortar kept on ice. The ratio of 1g of tissue to 2ml of buffer was used. Tobacco leaves were lightly dusted with Carborundum (600 mesh) and the sap inoculum applied with Q-tips.

F. BIOASSAY OF TSA INFECTIVITY

The local lesion host *Chenopodium amaranticolor* Coste & Reyn. was used for the bioassay of TSA infectivity. *Chenopodium* seeds were washed in cold running water for 2 hours, and germinated at 25°C on moist filter paper for 3 days. Seeds at the same stage of germination were then transferred to autoclaved 3:2:1 soil mix in plastic trays, and grown in a greenhouse at 22°C $\pm$ 2°C, 16 hours light period and 15,000 - 20,000 lux light intensity. After approximately 2 weeks these seedlings were planted individually in autoclaved 12 cm clay pots containing soil mix, and grown under the same conditions until they had developed 4 to 6 leaves. Plants with 4 fully developed leaves were selected for TSA infectivity assay. The inoculum was prepared from tobacco leaves as outlined in the previous section, E. The leaves of *C. amaranticolor* were dusted with Carborundum and the TSA inoculum applied with Q-tips using sufficient pressure to wet the whole leaf surface with the inoculum. Inoculated plants were incubated in a greenhouse at 18°C ± 2°C. Local lesions (Plate 2, E) developed on inoculated leaves in 6 or 7 days. Lesion counts were made 14 days after inoculation.

G. BIOASSAY OF TOBACCO NECROSIS VIRUS AS CONTAMINANT

Tobacco necrosis virus (TNV) is transmitted by *Olpidium*, and also produces local lesions on *C. amaranticolor* leaves (Plate 2, F). These lesions appear 3 to 4 days after sap inoculation. Leaves of Red Kidney bean, *Phaseolus vulgaris* L., were used to check for TNV contamination. This host plant is highly susceptible to TNV but

resistant to TSA, and thus can serve as an efficient differential indicator plant (Hiruki, 1967). Seeds were planted in University of California mixture (Baker, 1957) in clay pots and grown at 25°C. Seedlings, 12 to 20 days old, with 2 seed leaves were selected. Carborundum was dusted on the leaf surface and the inoculum applied with Q-tips. Inoculated plants were incubated at 18° ± 2°C, and the development of necrotic lesions in 3 days indicated the presence of TNV in the inoculum.

H. PREPARATION OF TISSUES FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

The following fixation, embedding and sectioning schedule was used for all tissues in this study unless stated otherwise in the text. Large pieces of tissues were cut into smaller pieces, approximately 2-3mm square.

a. Prefixed overnight at 4°C in 0.1M phosphate buffer pH 7.0 containing 2% glutaraldehyde and 2% formaldehyde.

b. Washed for 30 minutes in buffer, and then in distilled water.

c. Postfixed for 3 to 4 hours at room temperature in 2% aqueous osmium tetroxide.

d. Washed twice in water, 15 minutes for each wash.

e. Dehydrated through an ethanol series, 30 minutes in each of the following grades: 30, 50, 70, 80, 90, 95 and 100% - two changes in each of 95 and 100%.

f. Transferred tissues into propylene oxide, two changes of 15 minutes each.

g. Transferred tissues into 1:1 mixture of propylene oxide and an

Araldite mixture (27 parts Araldite : 23 parts dodecyl succinic anhydride, plus 2% DMP-30), and left them at room temperature for 36 hours.

h. Transferred tissues into rubber moulds and added the Araldite mixture. Left the embedded tissues at room temperature for 12 hours before incubating in an oven at 60°C for 36 hours to polymerise the Araldite mixture.

Sections were cut on a Reichert ultramicrotome using a diamond knife, and collected on formvar coated grids. Sections were stained in 2% aqueous uranyl acetate for 2 hours and post-stained in 0.2% aqueous lead citrate pH 13 (Venable and Coggleshall, 1965) for 4 minutes. Examinations were made with a Philips EM 200 at 60 kv. For calibration of the electron micrographs latex particles (109 nm diameter) were used.

I. SCANNING ELECTRON MICROSCOPY

Materials were fixed and dried according to the methods outlined in the text. Dried specimens were fixed to stubs using low resistance contact cement (Fullam Inc., New York, U.S.A.) and coated with 50Å of carbon and 50Å of gold in an Edwards Vacuum Evaporator. Coated specimens were examined with a Cambridge Stereoscan S4 operating at 20 kv and 15-30° tilt.

J. LIGHT AND FLUORESCENCE MICROSCOPY

Tissues were prepared according to the methods outlined in the text. Prepared specimens were examined with a Leitz Ortholux I

microscope, and observations were recorded on Kodak Plus X and Tri X film using a Leitz automatic camera.

For fluorescence microscopy live or fixed tissues were mounted on slides in a 0.01% solution of aniline blue fluorochrome in 1/15 M dibasic potassium phosphate pH 8.0. Observations were made with the Leitz Ortholux I microscope, fitted with a lamphousing 250 containing a 200W ultra-high pressure mercury lamp. The following combination of filters was used to give the maximum transmission in the wavelength range 350-370 nm: UV filter, 2mm UG1; UV + blue filter, 3mm BG3; red-suppression filter, 4mm BG38; and eyepiece barrier filter, K430. Under these conditions callose substances stained with aniline blue fluorochrome fluoresced a strong yellow-green colour (Eschrich and Currier, 1964).

CHAPTER IV

TRANSMISSION OF TOBACCO STUNT AGENT BY *OLPIDIUM* ZOOSPORES

A. INTRODUCTION

Since *Olpidium* zoospores are only able to acquire TSA *in vivo*, the association between TSA and stages of its vector is thought to be internal (Hiruki, 1965). Knowledge of the acquisition method is important in the examination of different stages in the life-cycle of *Olpidium* for visualisation of TSA. For the present study the mode of acquisition required confirmation, therefore experiments were set up to test whether acquisition occurs *in vivo* and/or *in vitro*. For subsequent experiments involving *Olpidium* transmission of TSA, it was necessary to determine the approximate concentration of *Olpidium*/TSA zoospores required for successful infection of tobacco with TSA. This would also provide an indication of whether all zoospores of *Olpidium*/TSA carry TSA. Hidaka *et al.* (1956) reported that symptom development of stunt occurred in tobacco plants grown in seedbeds before they were transplanted into the field. Large healthy plants transplanted into infested soil did not develop stunt symptoms. In the present study the effect of host plant age on transmission of TSA by *Olpidium*/TSA zoospores was also investigated.

B. *IN VIVO* ACQUISITION AND TRANSMISSION OF TSA BY *OLPIDIUM*

1. Materials and methods

a) *Olpidium* inoculation of TSA infected tobacco.

Seventy-two tobacco seedlings were grown in sand culture in 12 plastic trays at 18° ± 2°C. Half of these plants were inoculated with tobacco sap containing TSA when they were at the two leaves stage. The infectivity of TSA in this sap was assayed on leaves of *Chenopodium amaranticolor* (Chapter III). After incubating for 11 days, symptoms of stunt were observed on the inoculated tobacco, i.e. local chlorotic spots on the inoculated leaves and systemic vein-clearing on developing leaves. Half of these plants (18 plants in 3 trays) and half of the healthy controls were inoculated with a suspension of *Olpidium* zoospores (approximately 10^4 zoospores/ml) (Chapter III), by pipetting 5 ml of this suspension around the stem of each plant. Plants in the four treatment groups, i.e. (1) tobacco-TSA-*Olpidium*, (2) tobacco-TSA, (3) tobacco-*Olpidium*, (4) tobacco, were incubated for a further 11 days. The roots were then washed free of sand and observed with the light microscope for *Olpidium* infection, prior to incubating in 50 ml of distilled water to permit zoospore release. Twenty-five healthy tobacco seedlings (15 days old) were dip inoculated in each of the root extracts from the four treatments. These seedlings were transplanted into soil mix and grown for 3 weeks to observe any development of stunt symptoms. Leaf samples were taken from these plants and assayed for infectivity on leaves of *C. amaranticolor* and Red Kidney bean.

b) TSA inoculation of *Olpidium* infected tobacco.

The same experimental procedure was used as in a), except for the sequence of inoculations. Tobacco seedlings were inoculated

with *Olpidium*, and then after 5 days of incubation the leaves of half of these plants, and half of the controls, were inoculated with tobacco sap containing TSA. Infectivity of this sap inoculum was assayed on *C. amaranticolor*. Plants in the four treatment groups, i.e. (1) tobacco-*Olpidium*-TSA, (2) tobacco-*Olpidium*, (3) tobacco-TSA, and (4) tobacco, were incubated for 22 days to ensure development of systemic symptoms of stunt. Zoospore suspensions were prepared from the roots and used for dip inoculation of healthy tobacco seedlings. These seedlings were checked for stunt symptoms after a further 3 weeks, and assayed for infectivity on *C. amaranticolor* and Red Kidney bean.

2. Results

In both experimental procedures stunt transmission occurred only in the treatments involving both TSA and *Olpidium* inoculations. The initial inocula of tobacco sap containing TSA were found to be infective on *C. amaranticolor*. Likewise infectivity was only obtained from the treatments involving both TSA and *Olpidium*. No infectivity was obtained on leaves of Red Kidney bean. No stunt symptoms or TSA infectivity were observed with the three treatments which acted as controls.

Preliminary experiments on the TSA inoculation of *Olpidium* infected tobacco showed that it was necessary to inoculate the plants with sap within a week following the root inoculation with *Olpidium*. Also, incubation for a further 3 weeks was necessary in order to obtain consistent transmission of TSA.

C. IN VITRO ACQUISITION OF TSA BY *OLPIDIUM* ZOOSPORES

1. Materials and methods

Tobacco sap containing TSA was prepared by homogenising leaf tissue showing stunt symptoms in 0.01 M phosphate buffer pH 7.0, using a pre-cooled mortar and pestle. Buffer was added in the ratio of 5 ml buffer to 1 g leaf tissue. The homogenised tissues were squeezed through 4 layers of cheesecloth and the resulting sap, a 1:10 dilution, was kept on ice. More buffer was added to half of this sap to give a 1:20 dilution.

A suspension of *Olpidium* zoospores (10^5 zoospores/ml) was prepared as outlined in Chapter III. Five ml aliquots of this suspension were mixed with equal volumes of the 1:10 and 1:20 dilutions of sap. This gave preparations of zoospores (5×10^4/ml) respectively in 1:20 and 1:40 dilutions of sap. These preparations were assayed for infectivity on leaves of *C. amaranticolor* and Red Kidney bean. After incubating for 5 minutes at room temperature, these preparations were each used for the dip inoculation of 25, 2 weeks old, healthy tobacco seedlings. As a control treatment, tobacco seedlings were dip inoculated in 5 ml of the initial *Olpidium* zoospore suspension to which an equal volume of 0.01 M phosphate buffer had been added. All inoculated seedlings were transplanted into soil mix, and grown at 18° ± 2°C for 3 weeks to permit observation of the development of any stunt symptoms. After this incubation period the roots of plants from the treatments and control were examined for *Olpidium* infection. Leaf disc samples (40 per treatment) were taken randomly from each treatment group of tobacco, and assayed

for infectivity on *C. amaranticolor* and Red Kidney bean (Chapter III).

2. Results

The preparations of sap plus zoospores produced lesions on *C. amaranticolor*, but not on Red Kidney bean. The total lesion counts from 8 leaves of *C. amaranticolor* were as follows:

1:20 dilution of sap, plus zoospores - 217 lesions

1:40 dilution of sap, plus zoospores - 22 lesions

Olpidium infection was observed in the roots of tobacco plants from the treatment and control inoculations. No symptoms of TSA infection developed on any tobacco plants. Also, no infectivity was obtained from the leaf disc samples taken from the treatment and control inoculations of tobacco. Therefore, zoospores did not transmit TSA after contact with infectious TSA *in vitro*.

In preliminary experiments, zoospores lost their motility when the phosphate buffer concentration was above 0.01 M. Also the addition of 4-PTC (used to stabilise TSA infectivity) to the buffer resulted in the permanent loss of zoospore motility upon adding the sap to the zoospore suspension. The dilution of sap added to the zoospore suspension also influenced motility. Final sap dilutions lower than 1:20 resulted in loss of zoospore motility, and subsequently no *Olpidium* infection in the tobacco roots.

D. DETERMINATION OF *OLPIDIUM*/TSA ZOOSPORE CONCENTRATION FOR TRANSMISSION OF TSA

1. Materials and method

A concentrated suspension of *Olpidium*/TSA zoospores was

prepared (Chapter III), and diluted to give 25 ml of each of the following zoospore concentrations: 4×10^6, 4×10^5, 4×10^4, 4×10^3, 4×10^2, 40, and 4 zoospores/ml. Groups of 25, 2 weeks old, tobacco seedlings were dip inoculated with these different concentrations of zoospore suspensions. Inoculated seedlings were transplanted into soil mix and grown for 3 weeks at 18°± 2°C to observe the development of stunt symptoms. At the end of this incubation period the roots were examined for *Olpidium* infection.

This experiment was repeated using zoospore concentrations of 10^4, 10^3, 8×10^2, 4×10^2, 2×10^2, 10^2, 50, and 10 zoospores/ml.

2. Results

The results from these experiments are shown in Table 2.

TABLE 2. Effect of *Olpidium*/TSA zoospore concentration on transmission of tobacco stunt agent

Concentration of zoospores/ml	TSA infection*	*Olpidium* infection
4×10^6	25	+
4×10^5	25	+
4×10^4	25	+
4×10^3	25	+
4×10^2	9	+
40	0	+
4	0	-
1×10^4	25	+
1×10^3	25	+
8×10^2	22	+
4×10^2	11	+
2×10^2	9	+
1×10^2	0	+
50	0	+
10	0	-

*Number of tobacco plants showing stunt symptoms out of 25 plants inoculated.

A concentration of 200 to 400 *Olpidium*/TSA zoospores/ml was required for transmission of TSA. Infection by *Olpidium* occurred at concentrations which did not give symptoms of stunt infection.

E. EFFECT OF AGE OF TOBACCO PLANTS ON TRANSMISSION OF TSA BY *OLPIDIUM*/TSA ZOOSPORES

1. Materials and methods

Tobacco seeds were sown on vermiculite/soil mix in small plastic trays at intervals of 4 days up to 48 days from the first sowing, and the plants grown in the greenhouse at 18° ± 2°C. A suspension of *Olpidium*/TSA zoospores, approximately 10^4 zoospores/ml, was prepared as outlined in Chapter III. Each tray, ranging from 0 to 48 days after seeding, was then inoculated with *Olpidium*/TSA zoospores by pipetting 20 ml of the above zoospore suspension onto the soil. The plants were grown for a further 35 days to permit the development of any symptoms of TSA infection. At the end of this incubation period, symptoms of TSA infection were recorded. Also the roots were examined for *Olpidium* infection, and samples of leaf discs were taken from each treatment for infectivity assay on *C. amaranticolor* and Red Kidney bean (Chapter III).

2. Results

The infection of tobacco plants of different ages by *Olpidium* and TSA, following inoculation of the roots with zoospores of *Olpidium*/TSA, is shown in Table 3. Tobacco plants, from 4 to 48 days old at the time of inoculation, became infected with *Olpidium*/TSA.

However, stunt symptoms were only observed in plants 4 to 32 days old at the time of inoculation; and TSA infectivity was recovered from plants 4 to 28 days old when inoculated.

TABLE 3. Effect of tobacco plant age on infection by *Olpidium* and tobacco stunt agent

Age of tobacco when inoculated (days)	Root infection by *Olpidium*/TSA	Foliage symptoms of TSA infection	TSA infectivity
0	-	-	-
4	+	+	+
8	+	+	+
12	+	+	+
16	+	+	+
20	+	+	+
24	+	+	+
28	+	+	+
32	+	+	-
36	+	-	-
40	+	-	-
44	+	-	-
48	+	-	-

F. DISCUSSION

Olpidium zoospores transmitted TSA after contact *in vivo* but not *in vitro*. These results confirm previous reports (Hiruki, 1965, 1968). However, experiments on *in vitro* acquisition should be repeated when highly purified, infectious preparations of TSA are available. The use of such preparations would eliminate any possible influence of host components, present in crude sap, which may interfere with the acquisition of TSA by *Olpidium* zoospores. The absence of *in vitro* acquisition is further evidence that TSA is distinct from tobacco necrosis virus (TNV). *Olpidium* zoospores readily acquire TNV *in vitro* from partially purified TNV preparations

(Teakle, 1962). This mode of acquisition must be extremely sensitive, since lower concentrations of TNV can be detected by *Olpidium* acquisition *in vitro*, and subsequent transmission to an assay host, than by infectivity assay directly on leaves of the local lesion host, *Phaseolus vulgaris* L. (Fry and Campbell, 1966).

The results of experiments on the effect of zoospore concentration on TSA transmission suggest that only a partial population of *Olpidium*/TSA zoospores is capable of transmitting TSA. *Olpidium* infection in the roots of plants not showing stunt symptoms indicates that there is a low titre of TSA present, or that not all *Olpidium* zoospores carry TSA. A similar report was made for a mass culture of *Olpidium* carrying the lettuce big-vein agent (Lin *et al.*, 1970), which is also carried internally by its vector. Using single sporangium isolates, they demonstrated that the agent is not uniformly distributed among *Olpidium* thalli, even though the culture had been maintained on big-vein infected lettuce. The possible heterogeneity of *Olpidium* in its association with TSA must be considered when attempts are made to visualise the agent in different stages of the fungus.

The minimum zoospore concentration for TSA transmission was in the range of 200 to 400 zoospores/ml. Hidaka and Tagawa (1965) previously reported a minimum concentration of 800 zoospores/ml for TSA transmission. This variation in results may be due to the influence of such factors as environmental conditions on transmission, or to differences between isolates of *Olpidium*/TSA in their capacity to transmit TSA. In contrast to the minimum concentration of zoospores required for TNV transmission (Fry and Campbell, 1966), the

concentration required for TSA transmission is considerably higher. This may be due to the different modes of acquisition and disease agent-vector relationships.

The age of tobacco plants when inoculated with zoospores of *Olpidium*/TSA, affected the incidence of TSA infection. Plants up to 48 days old were infected by the fungus, but only those plants up to 32 days old developed symptoms of TSA infection. This suggests that tobacco plants, which were 4 to 5 weeks old, had developed resistance to TSA infection and multiplication. Hidaka *et al.* (1956) also concluded that tobacco plants less than 30 days old, when grown in infested soil, were susceptible to stunt, and that plants older than 30 days, when transplanted in infested soil, appeared to be resistant.

The results of these experiments on TSA transmission by zoospores suggest that TSA is only acquired *in vivo* by *Olpidium* zoospores, and that not all zoospores within a population carry the agent. Likewise, successful transmission is also influenced by the age of the host plant.

CHAPTER V

CHARACTERISATION OF TOBACCO STUNT AGENT

A. INTRODUCTION

The agent causing tobacco stunt disease was originally termed a virus on the evidence of graft transmission, symptoms of a virus disease in the infected host, and isolation of virus-like particles from infected tobacco (Hidaka *et al.*, 1956). The virus-like particles isolated were, however, non-infectious. Since that time transmission of TSA by mechanical inoculation with infectious sap (Hiruki, 1964), and by the fungus vector *O. brassicae* (Hiruki, 1965), have been demonstrated. No convincing evidence has been reported to establish the identity of this disease agent, but in the literature it is still accepted as a virus.

In the present study attempts have been made to isolate, characterise and visualise the infectious agent of tobacco stunt. The effects of certain factors on TSA infectivity in tobacco sap extracts were investigated to determine the optimal conditions for bioassay. Antibiotics and nucleases were added to the extraction buffer to provide an indication of the nature of the infectious agent. Likewise infected plants were treated with certain antibiotics to observe any remission of disease symptoms. This is known to occur with yellow diseases caused by Mollicutes-like organisms. The physical properties of TSA in tobacco sap were determined to provide

an indication of the stability of the agent for attempts at isolation of the infectious agent.

B. EFFECTS OF CERTAIN FACTORS ON THE EXPRESSION OF STUNT SYMPTOMS IN TOBACCO

1. Materials and methods

a) Effects of temperature

Twenty-one days old tobacco seedlings grown in vermiculite/soil mix at 25°C were dip inoculated with suspensions of *Olpidium* and *Olpidium*/TSA zoospores (Chapter III). Inoculated plants and healthy controls were transplanted into vermiculite/soil mix in small plastic trays (6 plants per tray), and grown at 25°C for 7 days to overcome any transplanting 'shock'. Each tray was given a number according to the inoculation treatment, i.e.

trays 1 to 9: tobacco-*Olpidium*/TSA

trays 10 to 18: tobacco-*Olpidium*

trays 19 to 27: tobacco

The plants were incubated in growth chambers, with 5,000-10,000 lux light intensity, for 14 days at different temperatures (Table 4). After this period, 30 leaf disc samples (approx. 1g) from the plants in each tray were bioassayed for TSA infectivity on *Chenopodium amaranticolor* and on Red Kidney bean to monitor any TNV contamination (Chapter III). The tobacco plants were incubated for a further 14 days after transferring some of the trays to different temperatures (Table 4). Leaf samples were also harvested at the end of this incubation period and bioassayed for TSA infectivity. The development

of stunt symptoms and lesions on *C. amaranticolor* were recorded.

TABLE 4. Incubation temperatures for trays containing healthy, *Olpidium*-inoculated and *Olpidium*/TSA-inoculated tobacco

Period of incubation (days)	Incubation temperature								
	17°C			25°C			33°C		
0 — 14	*1	*2	*3	4	5	6	7	8	9
	10	11	12	13	14	15	16	17	18
	19	20	21	22	23	24	25	26	27
15 — 28	*1	*5	8	4	2	9	7	3	6
	10	14	17	13	11	18	16	12	15
	19	23	26	22	20	27	25	21	24

Numbers in the table indicate individual tray numbers

* = Plants developed symptoms of stunt and lesions developed on *C. amaranticolor*

b) Effects of antibiotics

(i) Tobacco seedlings were grown from seed in vermiculite/soil mix at 25°C for 10 days and then at 18° ± 2°C for 10 days. The roots were washed free of soil and then dip inoculated for 1 hour with suspensions of *Olpidium* and *Olpidium*/TSA zoospores. After inoculation the roots were washed, and the seedlings incubated in a half strength Hoagland's solution for 4 days. The roots were examined for infection by the fungus, and then 10 seedlings from each treatment, i.e. healthy, *Olpidium*-inoculated, and *Olpidium*/TSA inoculated, were incubated in the following antibiotic solutions for 24 hours: 10, 100 & 1000 ppm tetracycline; 1000, 10000 & 100000 I.U. penicillin; 10, 100 & 1000 ppm

chloramphenicol. Distilled water was used as a control. After treatment with antibiotics the seedlings were transplanted into vermiculite/soil mix and grown at 18° ± 2°C for 6 weeks. The development of any symptoms of stunt infection was recorded.

(ii) Ten days old tobacco seedlings were dip-inoculated for 1 hour in suspensions of *Olpidium* and *Olpidium*/TSA zoospores. These inoculated seedlings and healthy controls were transplanted into vermiculite/soil mix and grown for 25 days to permit the development of any symptoms of stunt infection. At the end of this period, soil was washed from the roots of plants in each treatment, and 10 seedlings incubated for 24 hours in solutions of the same antibiotics as in section (i). After treatment with antibiotics, the seedlings were transplanted and grown as in section (i). The development of any symptoms of stunt infection was recorded.

2. Results

a) Effects of temperature

Only tobacco plants inoculated with *Olpidium*/TSA zoospores and grown at 17°C developed symptoms of stunt during the first incubation period of 14 days. Infectivity of TSA was obtained from these plants (Table 4). No lesions developed on Red Kidney bean leaves. Low temperature had an effect on the growth of both *Olpidium*-inoculated and healthy control plants. These plants were smaller in size and the leaves were lighter in colour in contrast with plants grown at 25° and 33°C. During the second incubation period of 14 days only plants in tray numbers 1 and 5 had symptoms of stunt, and associated TSA infectivity. Tray No. 1 had been maintained for 28 days at 17°C,

whereas tray No. 5 was transferred from 25°C to 17°C after the first incubation period. Symptoms were masked at 25°C, but developed when the plants were incubated at 17°C. The plants grown at 33°C, when put at 17°C, did not develop any stunt symptoms and no infectivity was obtained from these plants (Plate 1, D & E).

b) Effects of antibiotics

The development of stunt symptoms in tobacco was not suppressed by treatments including 10 to 1,000 ppm tetracycline, 10 to 1,000 ppm chloramphenicol, and 1,000 to 100,000 I.U. penicillin. The highest concentration of each antibiotic used caused phytotoxicity to different extents in the treated plants. The time of application of the antibiotics did not have any effect on the development of stunt symptoms in tobacco inoculated with *Olpidium*/TSA.

C. EFFECTS OF CERTAIN FACTORS ON THE BIOASSAY OF TSA IN TOBACCO SAP

1. Materials and methods

a) Buffer pH

One leaf disc was taken from each of 30 tobacco leaves showing systemic necrosis 14 days following sap inoculation of the plants with TSA. Each sample of 30 leaf discs (approximately 1 g) was homogenized in a precooled mortar and pestle with 2 ml of phosphate/PTC buffer. The buffer pH was varied in the range of pH 5.0 to 8.5 for different leaf disc samples. Each sap inoculum was assayed for TSA infectivity on 8 half leaves of *C. amaranticolor* dusted with 600 mesh Carborundum. The inoculated leaves were rinsed with water after inoculation and the plants were incubated at 18° ± 2°C. Lesion counts were made after

14 days of incubation.

In this experiment and subsequent bioassays for TSA infectivity precautions were taken to exclude variation in the susceptibility of different leaves and plants of *C. amaranticolor* to TSA infection. Plants with 4 leaves were selected at a uniform stage of development, and each inoculum was applied to the 4 leaf positions on different plants. When comparing only two inocula, these inocula were applied to the two halves of the same leaves. When bioassaying for TSA infectivity, inoculations were also made on leaves of Red Kidney bean for an indication of any TNV as contaminant.

b) Buffer molarity

Samples of 30 leaf discs, as in section a), were homogenised with 2 ml of 0.001 M 4-PTC prepared in different molar concentrations of phosphate buffer pH 7.0 ranging from 0.001 M to 0.5 M. The different inocula were bioassayed for TSA infectivity on leaves of *C. amaranticolor*.

c) Incubation temperature

Two g of tobacco leaves, showing systemic necrosis 14 days after sap inoculation with TSA, were homogenised with 18 ml of phosphate/PTC buffer, and the resulting sap extract kept on ice. This inoculum was used to inoculate four leaves on each of 16 *C. amaranticolor* plants. Following inoculation 4 plants were incubated at each of the following temperatures: 17°, 21°, 25° and 33°C. Lesion counts were made from 4 to 16 days after inoculation.

2. Results

a) Buffer pH

The lesion counts obtained from 2 experiments on the effect of buffer pH on TSA infectivity in tobacco sap are listed in Table 5 (Appendix). These results have also been plotted on a graph (Figure 1). The highest infectivity was obtained with buffer in the range of pH 6.5 to 7.5. In contrast, buffer at pH 5.0 reduced the level of TSA infectivity recovered from tobacco sap.

b) Buffer molarity

Buffer of high molarity decreased the infectivity of TSA in tobacco sap. The optimal molarity was 0.01 (Figure 2). Buffers with molarity higher than 0.05 reduced the TSA infectivity considerably. Inocula prepared with phosphate buffer or with distilled water, both without 4-PTC, contained much less infectivity than those prepared with 0.01 M phosphate /PTC. The lesion counts obtained from 2 experiments are listed in Tables 6 & 7 (Appendix).

c) Incubation temperature

The effect of incubation temperature on the development of local lesions on *C. amaranticolor* leaves following sap inoculation with TSA is shown in Figure 3. At 21° and 25°C the lesions appeared first after 5 days of incubation (day 5), whereas at 17°C only a few lesions had appeared by day 6. The highest number of lesions was produced at 17° and 21°C. This number was reached by day 12 at 21°C, but not until day 16 at 17°C. At 25°C lesion development had reached its maximum by day 14, but the lesion count was only approximately half of the highest counts at 17° and 21°C. No lesions developed at 33°C. The lesion counts obtained from plants incubated

FIGURE 1. Effect of buffer pH on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

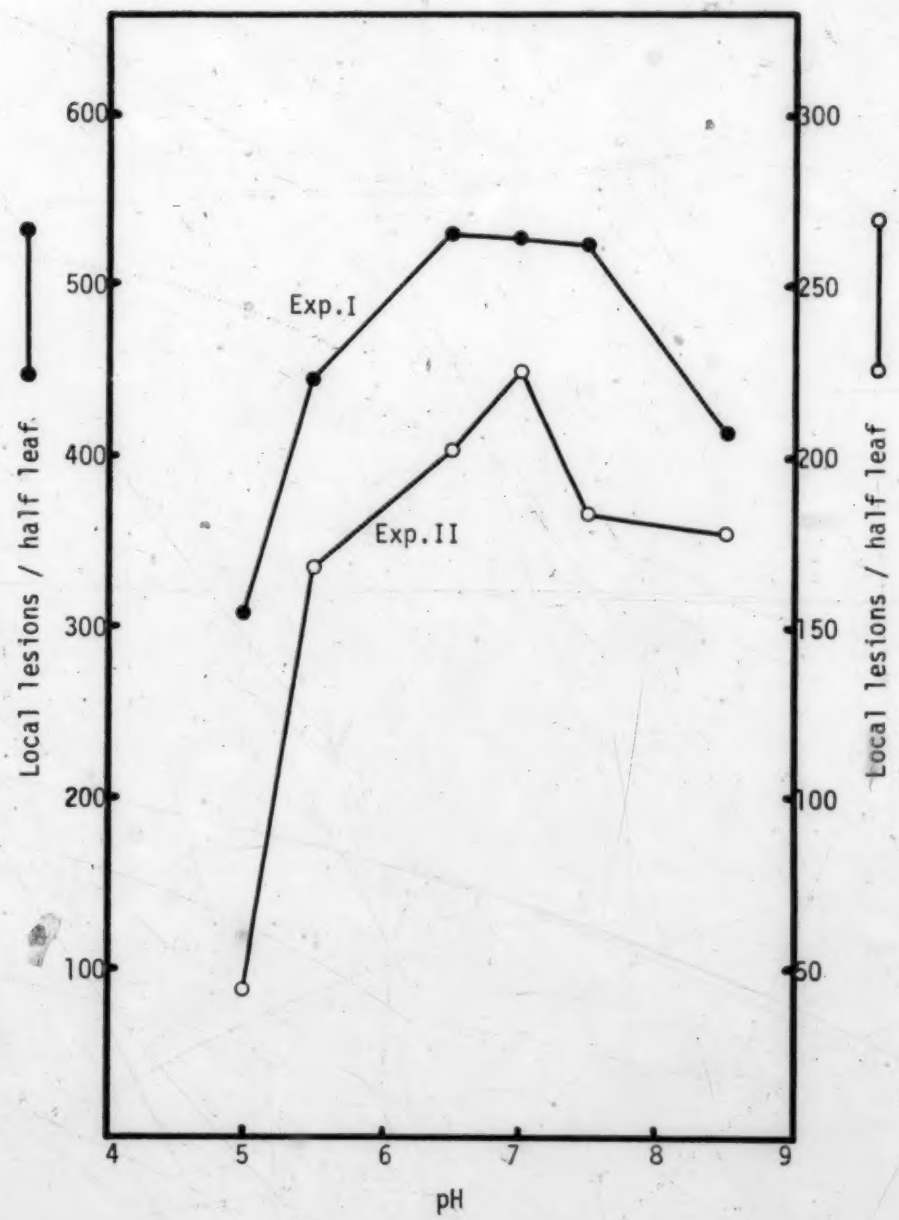
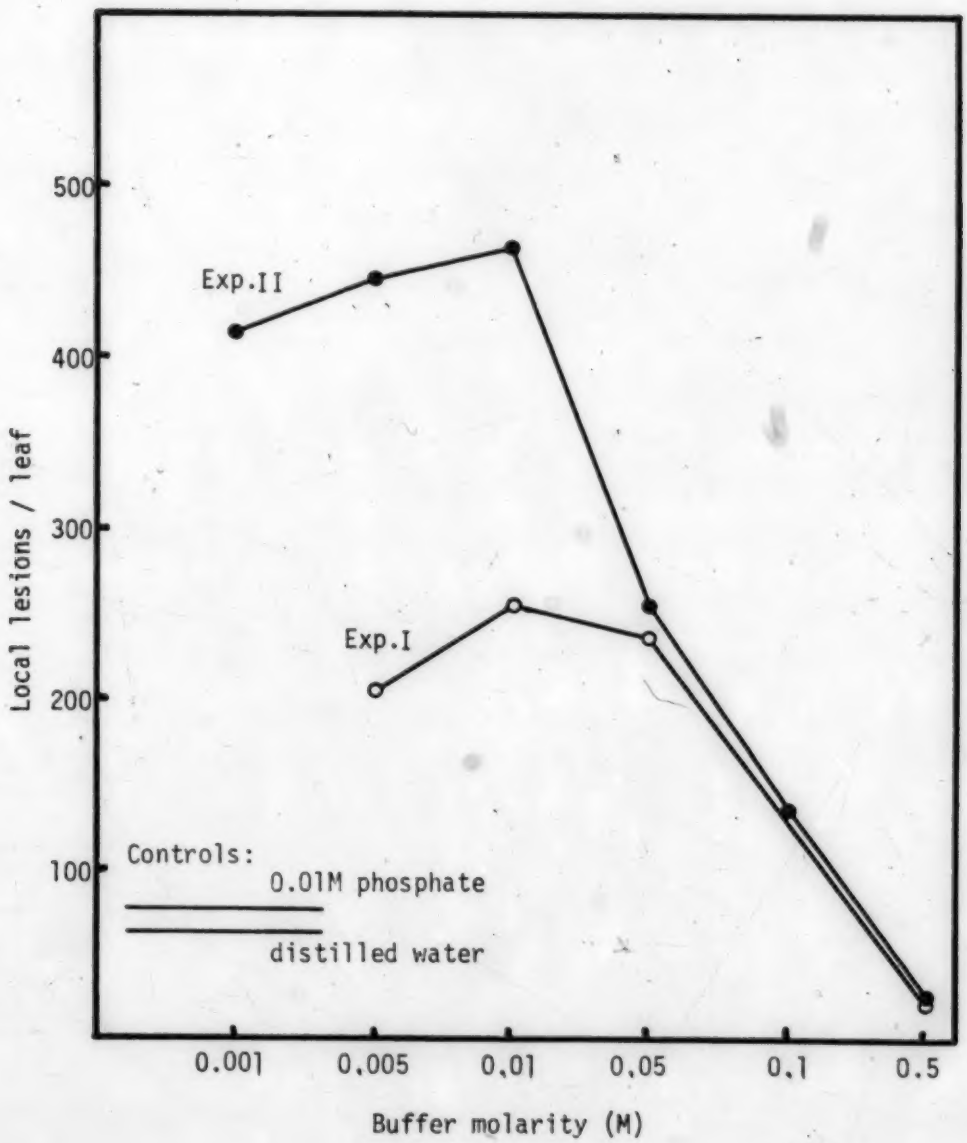


FIGURE 2. Effect of buffer molarity on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*



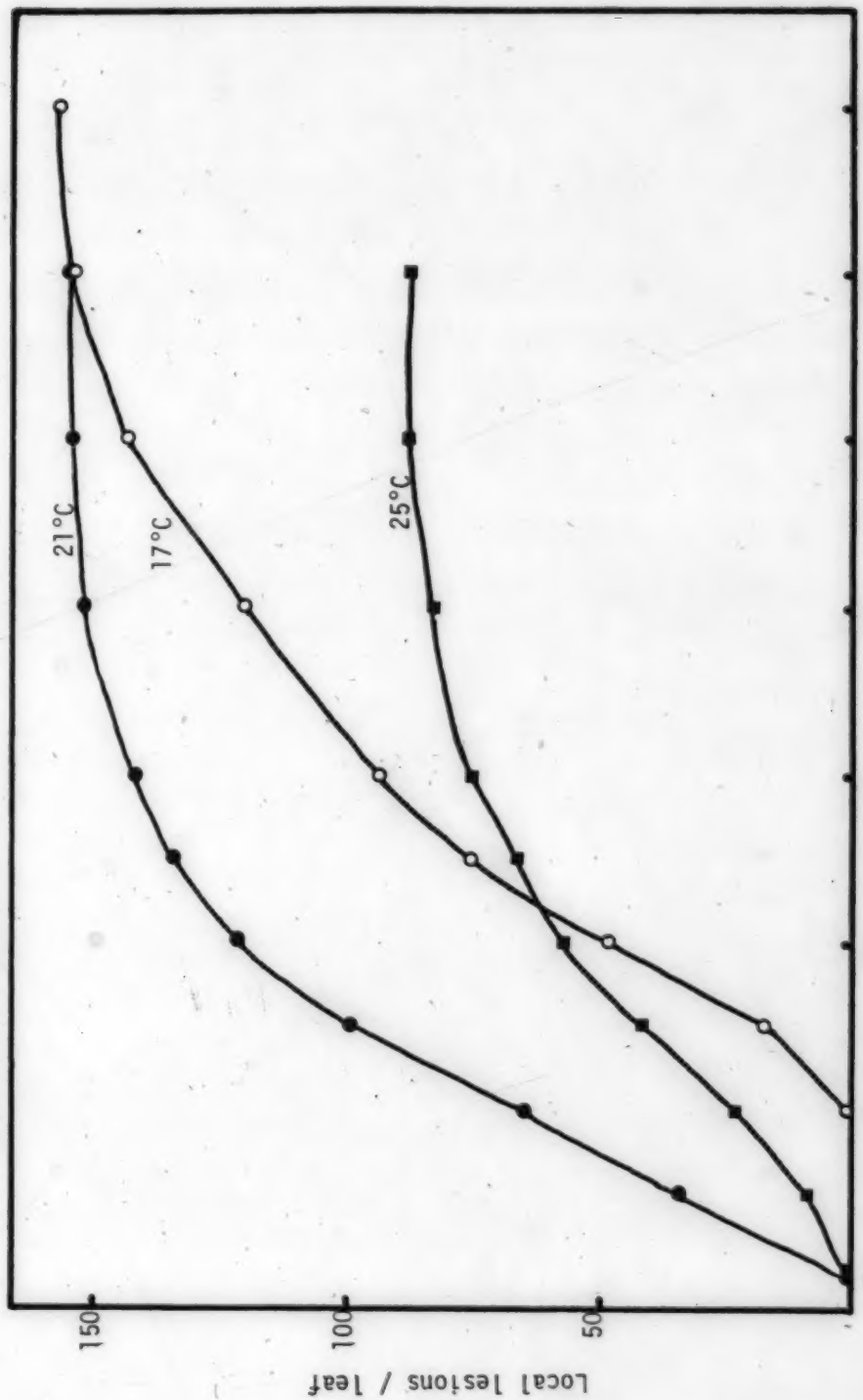


FIGURE 3. Effect of incubation temperature on tobacco stunt agent lesion number on *Chenopodium amaranticolor*

at 17°, 21° and 25°C are listed on Tables 8, 9 & 10 (Appendix).

D. INCREMENT CURVE OF TSA INFECTIVITY FROM TOBACCO AS DETERMINED BY BIOASSAY

1. Materials and methods

a) Increment curve obtained from tobacco inoculated with *Olpidium*/TSA

The roots of 21 days old tobacco seedlings grown in vermiculite/soil mix were washed free of soil and then dip inoculated with a suspension of *Olpidium*/TSA zoospores for 1 hour (Chapter III). The inoculated seedlings were transplanted into vermiculite/soil mix and grown at 18° ± 2°C for 6 weeks. Samples of 30 leaf discs (approximately 1 g) were taken at intervals of 2 days from 10 to 36 days after inoculation of the roots with zoospores, and bioassayed for TSA infectivity. The leaf samples were homogenised with 2 ml phosphate/PTC buffer, and assayed on 8 leaves of *C. amaranticolor*. Lesion counts were made after incubating for 14 days.

b) Increment curve obtained from tobacco inoculated with sap containing TSA

Healthy tobacco seedlings were grown for one month in vermiculite/soil mix in small plastic trays (Chapter III). The leaves were dusted with Carborundum and inoculated with a sap sample obtained from infected tobacco plants. The inoculum was prepared by homogenising 4 g tobacco leaves, showing systemic necrosis 16 days after sap inoculation with TSA, with 20 ml phosphate / PTC buffer. Inoculated plants were grown at 18° ± 2°C, and samples of 30 leaf discs

(approximately 1g) were harvested from inoculated leaves and from non-inoculated leaves at intervals of 2 days from 4 to 24 days after inoculation. These leaf samples were bioassayed for TSA infectivity as outlined in a). The inocula were applied to half leaves of *C. amaranticolor*. Lesion counts were made after incubation for 14 days.

2. Results

a) Increment curve obtained from tobacco inoculated with *Olpidium*/TSA

Infectivity of TSA in tobacco leaves was first detected 14 days after inoculation of the roots with zoospores (Figure 4). Symptoms of stunt were observed on the tobacco plants at 20 days after inoculation, and the highest infectivity was obtained at day 22. At day 30 symptoms of stunt were well developed but the infectivity was low. No lesions developed on Red Kidney bean. In both experiments the highest infectivity was obtained at the beginning of stunt symptom development on tobacco. As the symptoms became more severe, the infectivity dropped. The lesion counts obtained are listed in Table 11 (Appendix).

b) Increment curve obtained from tobacco inoculated with TSA in sap

Infectivity of TSA was recovered from inoculated tobacco leaves 4 days after inoculation. The highest infectivity was obtained 12 to 14 days after inoculation (Figure 5). Symptoms first appeared on the inoculated leaves at day 6, and by day 14 the necrosis was severe. After day 14 there was a decrease in TSA infectivity. Infectivity was obtained from non-inoculated leaves after 10 to 12 days after inoculation of the plants. Symptoms first developed on these leaves on days 12 to 14. The highest infectivity was obtained on days 16 to 18. Stunting of plants

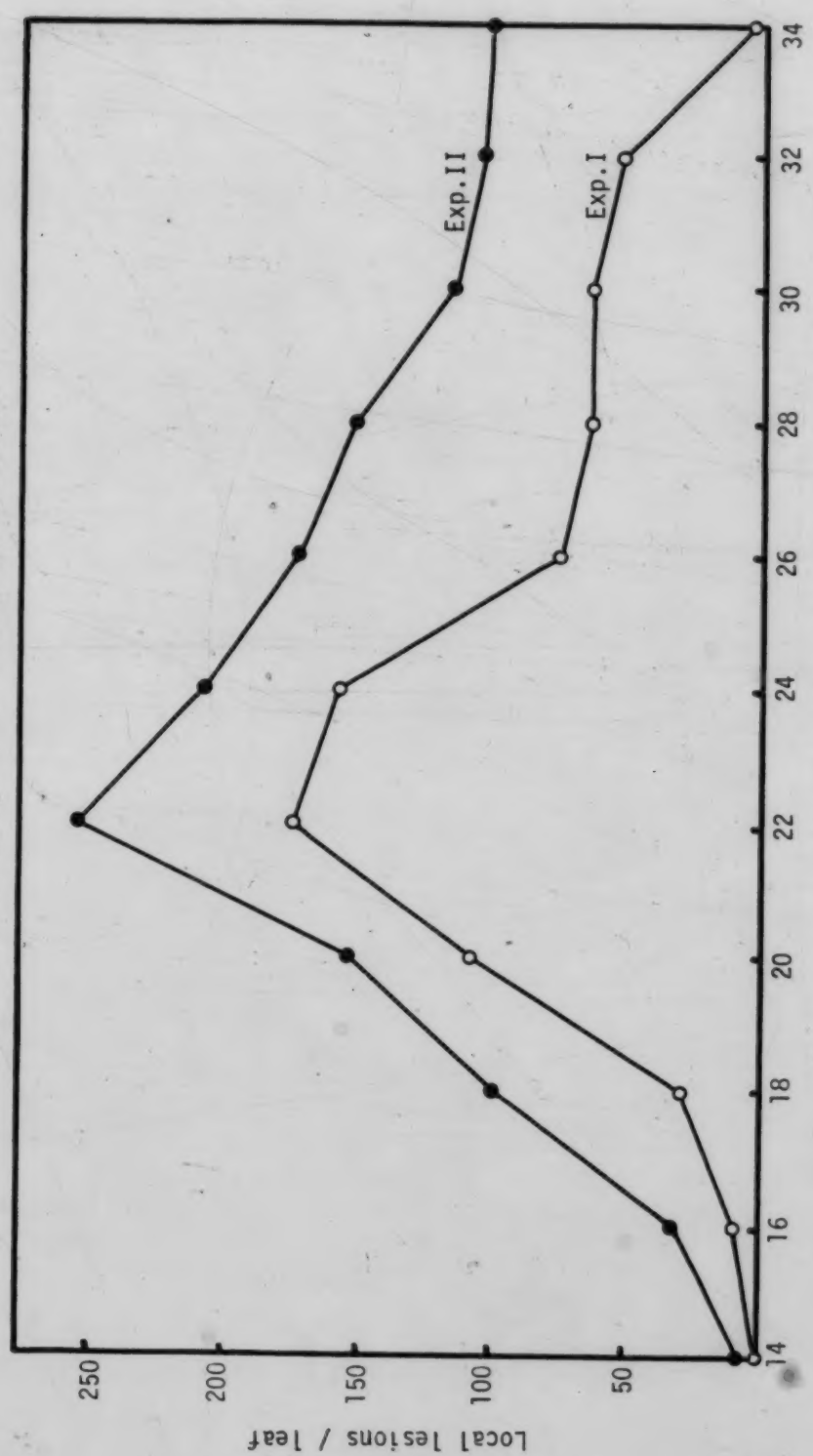


FIGURE 4. Increment curves of tobacco stunt agent infectivity bioassayed on *Chenopodium amaranticolor* following *Olpidium* transmission to tobacco

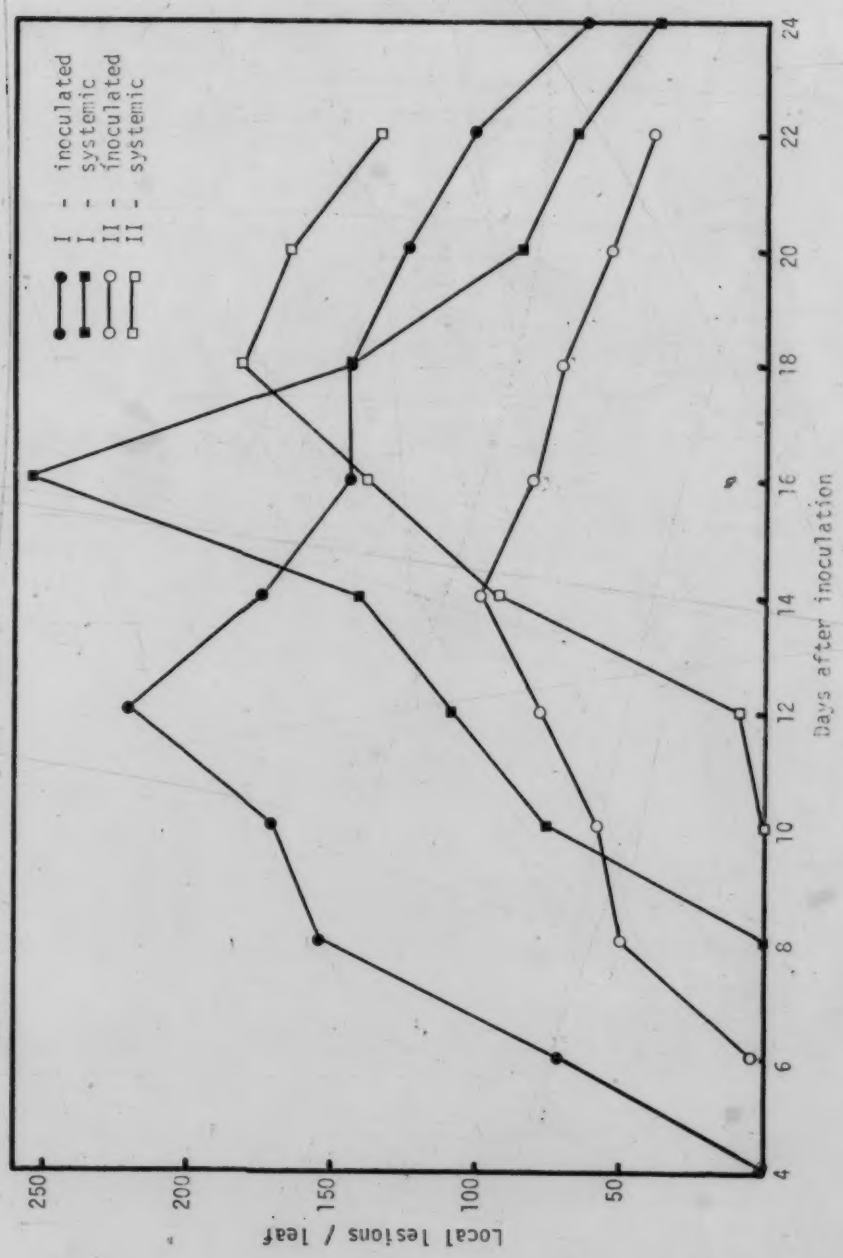


FIGURE 5. Increment curves of tobacco stunt agent infectivity bioassayed on *Chenopodium amaranticolor* following sap inoculation of tobacco

occurred after day 20. The recovery of TSA infectivity from systemically infected leaves was possible 6 days after the first detection of TSA in inoculated leaves. Highest infectivity in all leaves was obtained prior to symptoms of necrosis and stunting becoming severe. Lesion counts obtained are listed in Tables 12 & 13 (Appendix).

E. EFFECTS OF CERTAIN ADDITIVES ON TSA INFECTIVITY IN TOBACCO SAP

1. Materials and methods

a) Anti-oxidants

Two leaves of tobacco showing symptoms of systemic necrosis were harvested 16 days after sap inoculation of the plants with TSA. These leaves were cut into half exactly down the mid-veins. Two half leaves, one from each leaf, were homogenised with phosphate/PTC buffer as a control, and TSA infectivity was bioassayed on one half of leaves of *C. amaranticolor*. The other 2 half leaves of infected tobacco were homogenised with phosphate/PTC buffer containing 1% sodium sulphite, and inoculated to the remaining half of the *C. amaranticolor* leaves. The same procedure was repeated for testing the effect of 1% ascorbic acid added to the phosphate/PTC buffer. Lesion counts were made after incubating for 14 days.

b) Antibiotics

The same procedure as outlined in a) was used for the preparation and bioassay of inocula to determine the effect of adding antibiotics to the phosphate/4-PTC buffer. The following solutions were tested: phosphate/PTC + 1,000 ppm tetracycline, phosphate/PTC + 1,000 ppm

chloramphenicol, and phosphate/PTC +10,000 I.U. penicillin. The sap extracts were incubated at 20°C for 10 minutes prior to the bioassay for TSA infectivity.

c) Nucleases and protease

The same procedure was used for the preparation and bioassay of inocula as outlined in a). The following nuclease and protease solutions were tested: phosphate/PTC + 5 µg ribonuclease (RNase) per ml, phosphate/PTC + 100 µg deoxyribonuclease (DNase) per ml, and phosphate/PTC + protease at concentrations of 500 µg, 1,000 µg and 2,000 µg per ml.

The effect of RNase concentration on TSA infectivity in tobacco sap was also determined. Eight g of tobacco leaves showing systemic necrosis, 16 days after sap inoculation of plants with TSA, were homogenised with 8 ml 0.02 M phosphate pH 7.0 + 0.001 M 4-PTC. Solutions of 0.02, 0.2, 2 and 20 µg RNase/ml were prepared in distilled water. Equal volumes of the sap extract in phosphate/PTC buffer and the RNase preparations were mixed together and incubated at 20°C for 10 minutes. Thus the final concentrations of RNase obtained were 0.01, 0.1, 1 and 10 µg/ml. After incubation, these different preparations of sap plus RNase were bioassayed for TSA infectivity on *C. amaranticolor*. A control inoculum consisting of sap in phosphate/PTC buffer mixed with an equal volume of distilled water was also bioassayed.

The effect of RNase on TSA infectivity in tobacco sap over a period of incubation at 20°C was determined. Tobacco sap extracted in 0.02 M phosphate/PTC buffer was prepared as above, and samples were mixed with equal volumes of RNase in distilled water to give final concentrations of 0.001 and 0.01 µg RNase/ml. A control inoculum was prepared by adding an equal volume of water to the sap

extract in phosphate/PTC buffer. These inocula were bioassayed for TSA infectivity after incubating at 20°C for 0, 10, 20, 30, 40 and 60 minutes.

d) Nuclease inhibitors

Crude bentonite was purified as outlined for use in virus nucleoprotein extraction (Dunn and Hitchborn, 1965) and in nucleic acid extraction (Fraenkel-Conrat et al., 1961). The former procedure involved suspending the final bentonite pellet in phosphate buffer containing magnesium sulphate. This sample is hereafter referred to as Mg-bentonite in order to distinguish it from the sample obtained from the second procedure. The effects of Mg-bentonite and bentonite on TSA infectivity were tested using the procedure outlined in a). The following concentrations were used: 2.5, 5.0 and 12.0 mg Mg-bentonite per ml, and 3.0, 8.0 and 16.0 mg bentonite per ml.

The effect of different concentrations of yeast RNA on TSA infectivity in tobacco sap was investigated. Fifteen g of tobacco leaves showing early systemic symptoms, 16 days after sap inoculation of the plants with TSA, were homogenised with 15 ml 0.02 M phosphate/PTC buffer pH 7.0. Samples of this sap extract were mixed with equal volumes of yeast RNA prepared at the following concentrations in distilled water: 0.2, 2, 10, 20 and 200 mg/ml. After mixing the preparations contained yeast RNA at 0.1, 1, 5, 10 and 100 mg/ml. These preparations were incubated for 1 hour at 20°C and then bioassayed for TSA infectivity on leaves of *C. amaranticolor*.

The effects of yeast RNA and Mg^{2+} on TSA infectivity in tobacco sap over a period of incubation for 1 hour at 20°C were determined. Ten g of tobacco leaves showing early systemic symptoms

were homogenised with 10 ml of 0.02 M phosphate/ PTC buffer. Samples of this extract were mixed with equal volumes of yeast RNA and magnesium chloride in distilled water to give final concentrations of 5 mg yeast RNA per ml and 0.01 M Mg^{2+}. These mixtures were bioassayed for TSA infectivity after incubating for 0, 10, 20, 30 and 60 minutes at 20°C.

2. Results

a) Anti-oxidants

Addition of 1% sodium sulphite and 1% ascorbic acid caused a reduction in TSA infectivity (Figure 6). Buffer containing ascorbic acid was pH 4.5, whereas the buffer plus sodium sulphite was pH 7.5. The latter had less adverse effect on TSA infectivity in tobacco sap. Lesion counts are listed in Table 14 (Appendix).

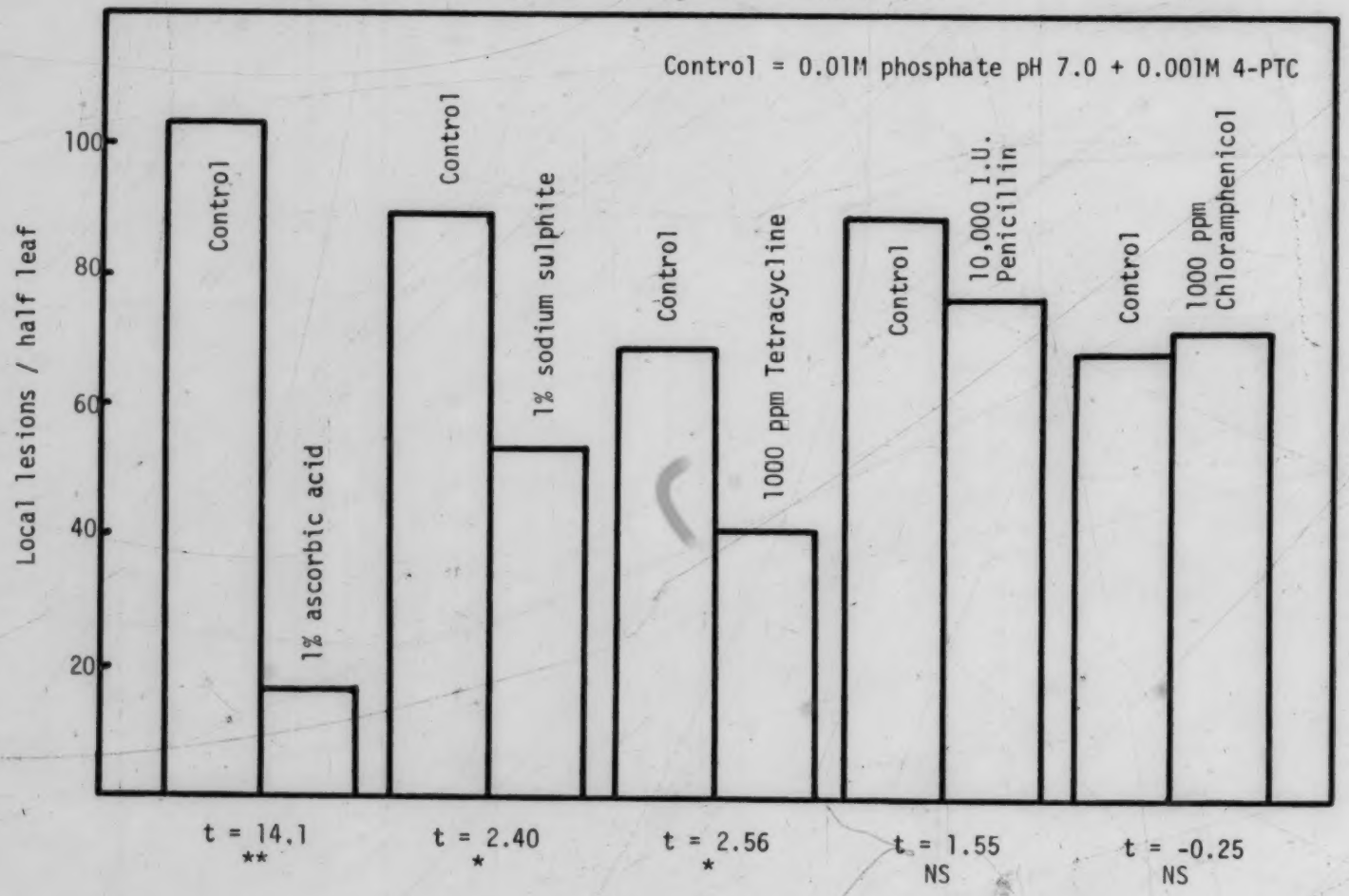
b) Antibiotics

Chloramphenicol and penicillin at the concentrations tested had no significant effect on TSA infectivity in tobacco sap (Figure 6). However, the addition of tetracycline caused a reduction in TSA infectivity significant at the 5% level. The lesion counts are listed in Table 14 (Appendix).

c) Nucleases and protease

There was no effect of DNase at a concentration of 100 µg/ml on TSA infectivity in tobacco sap. However, infectivity was completely inactivated by 5 µg/ml RNase (Figure 7). Protease at a concentration of 500 µg/ml reduced infectivity by approximately 50%. Concentrations of 1,000 and 2,000 µg/ml were almost completely effective in removing infectivity. Lesion counts are listed in Table 15 (Appendix).

FIGURE 6. Effects of anti-oxidants and antibiotics on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*



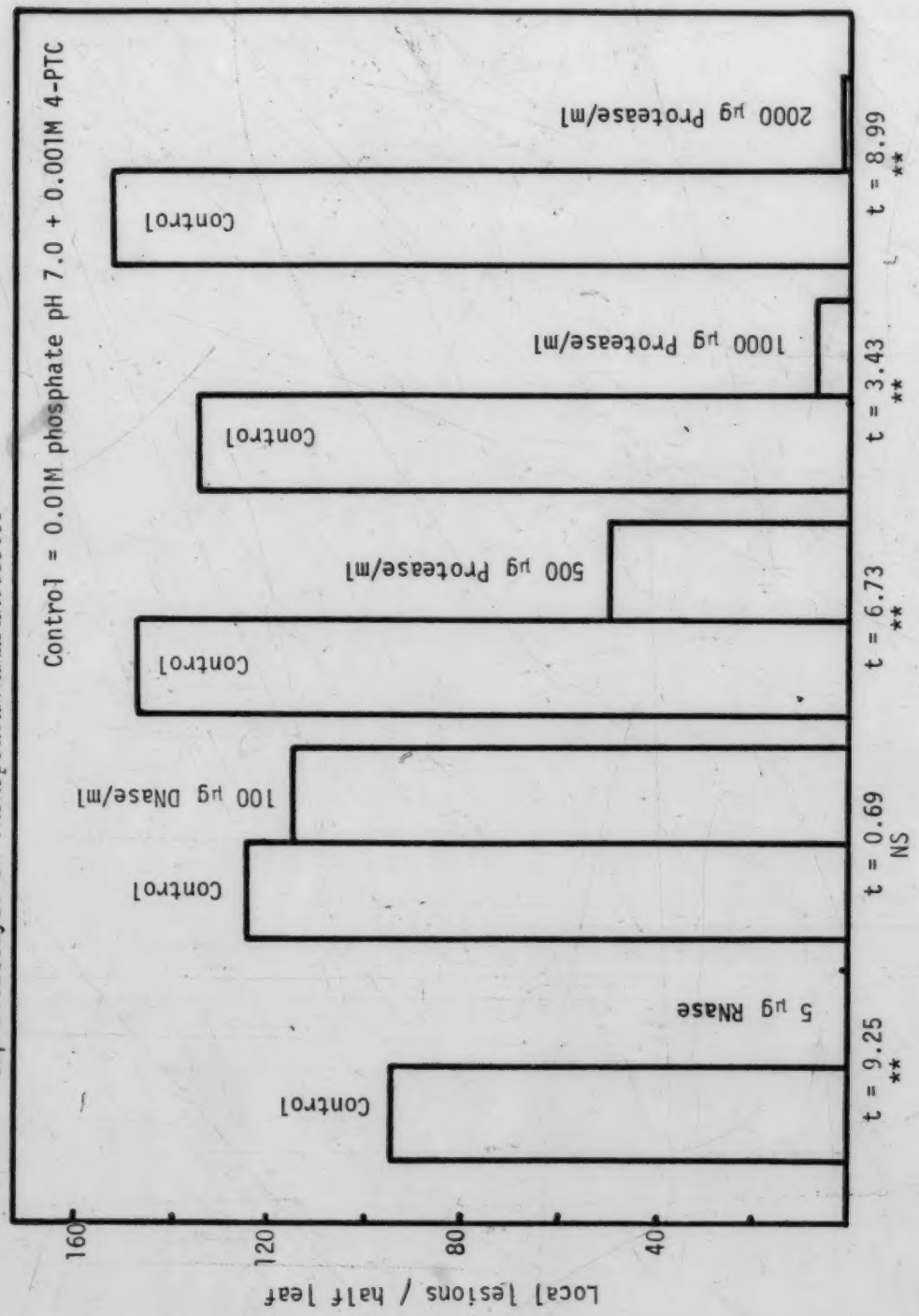


FIGURE 7. Effects of RNase, DNase and protease on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amanticolor*

Ribonuclease at concentrations higher than 0.1 µg/ml completely inactivated TSA infectivity in the sap preparations (Figure 8). At a concentration of 0.0001 µg RNase/ml, infectivity of sap extracts was the same as for control preparations. Lesion counts are listed in Table 16 (Appendix).

The kinetics of RNase action at concentrations of 0.001 and 0.01 µg/ml on TSA infectivity in tobacco sap is illustrated in Figure 9. Infectivity in the control preparations decreased considerably over the total incubation period of 1 hour. However, a greater loss of infectivity during the first 10 minutes of incubation was evident in the presence of added RNase. Even during the time to make the inoculations at 0 minutes incubation, the infectivity of the sap extracts containing RNase was reduced by approximately 50% in contrast with the controls. However, after 10 minutes of incubation the infectivity in extracts containing RNase did not decrease so rapidly. The difference between the infectivity of treatment and control extracts after 60 minutes of incubation was less evident. Lesion counts are listed in Table 17 (Appendix).

d) Nuclease inhibitors

Mg-bentonite at concentrations of 2.5 and 5.0 mg/ml had no effect on TSA infectivity. However, at 12 mg/ml TSA infectivity was completely inactivated (Figure 10). Bentonite at 3 mg/ml reduced infectivity by approximately 50%. At 8 mg/ml or higher concentrations, infectivity was extremely low or completely inactivated. Lesion counts are listed in Table 18 (Appendix).

The addition of yeast RNA at concentrations of 5 and 10 mg/ml gave the highest TSA infectivity in tobacco sap incubated for 1 hour

FIGURE 8. Effect of RNase concentration on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

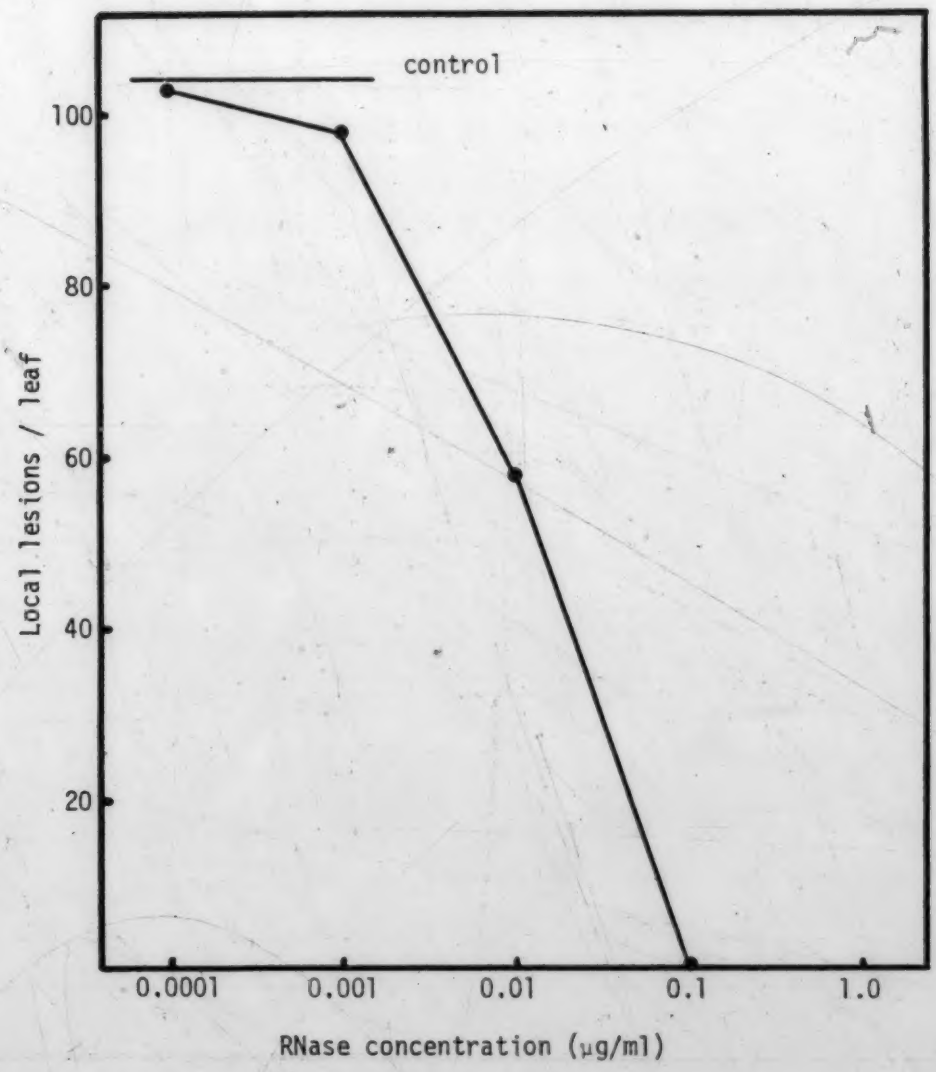


FIGURE 9. Kinetics of RNase treatment on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

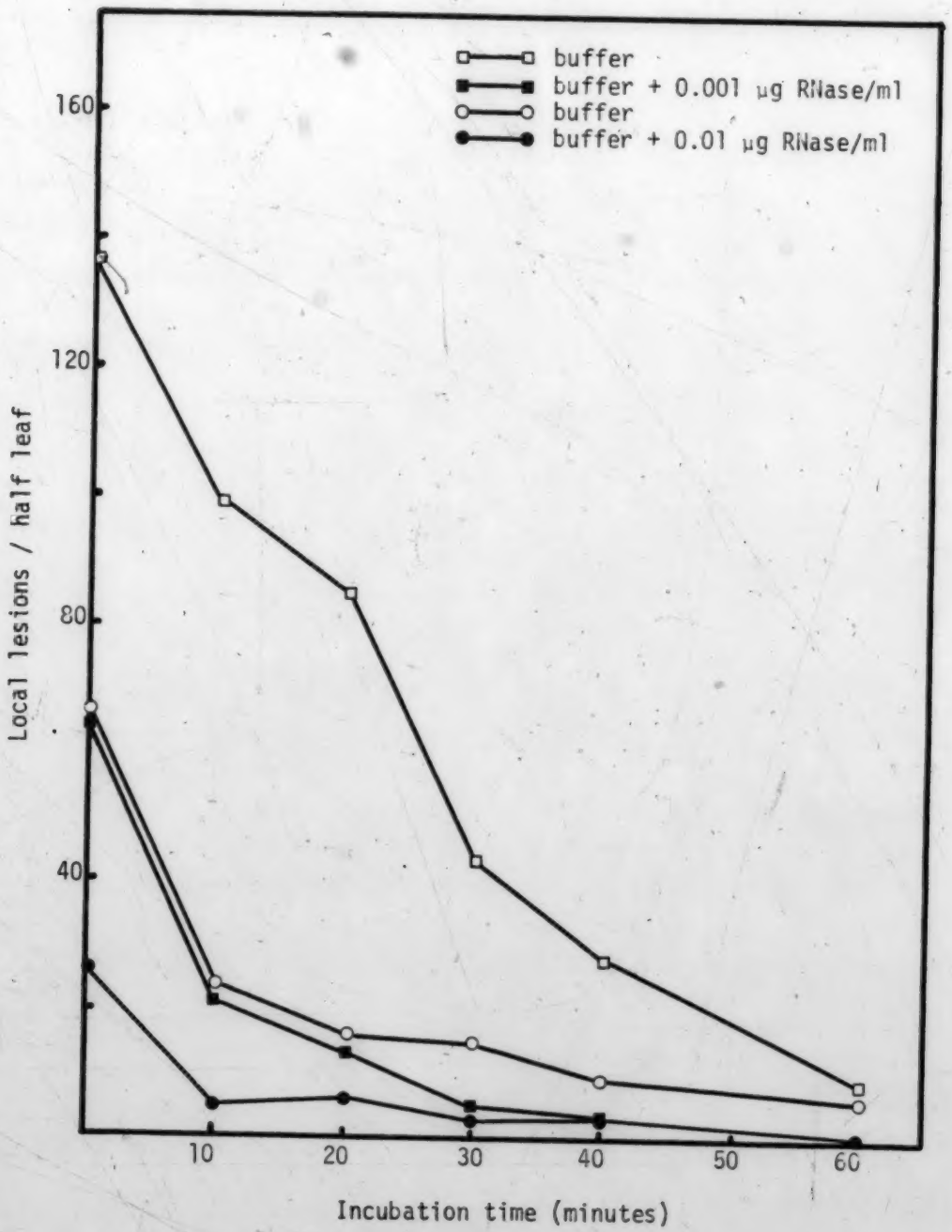
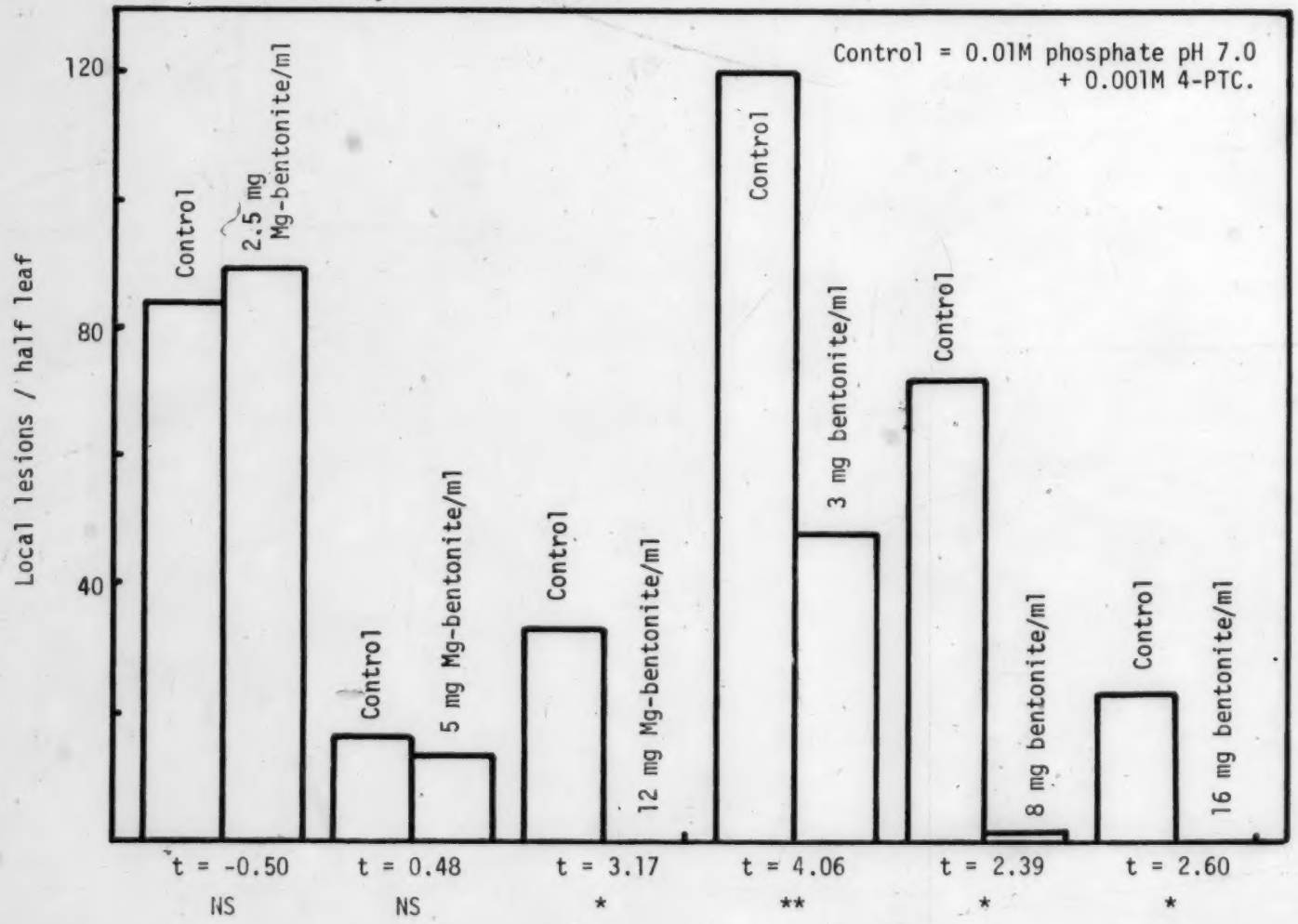


FIGURE 10. Effect of bentonite on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*



at 20°C (Figure 11). Lesion counts are listed in Table 19 (Appendix).

Yeast RNA at the concentration of 5 mg/ml partly stabilised TSA infectivity in tobacco sap. This effect occurred even during the time taken to make the inoculations at 0 minutes incubation time. Inactivation occurred during the 1 hour of incubation, but a higher level of infectivity was obtained from the preparation including yeast RNA than from the control (Figure 12). Addition of Mg^{2+} at 0.01 M did not stabilise TSA infectivity. Over the total incubation period of 1 hour, inactivation occurred at the same rate in both Mg^{2+} treated preparations and in the control (Figure 12). Lesion counts are listed in Table 20 (Appendix).

F. PHYSICAL PROPERTIES OF TSA IN TOBACCO SAP

1. Materials and methods

a) Dilution end point

Five g of tobacco leaves showing early systemic symptoms, 16 days following sap inoculation with TSA, were homogenised with 5 ml phosphate/PTC buffer, and the sap squeezed through cheesecloth. This sap was considered as a 1:1 dilution. The sap was kept on ice and further diluted with buffer to make 1:5, 1:10, 1:50 and 1:100 dilutions. These preparations were bioassayed for TSA infectivity on leaves of *C. amaranticolor*.

b) Longevity

An extract of tobacco sap containing TSA was prepared by homogenising 10 g of leaves, showing early systemic symptoms, with 10 ml phosphate/PTC buffer pH 7.0. Another extract was prepared using

FIGURE 11. Effect of the addition of yeast RNA on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

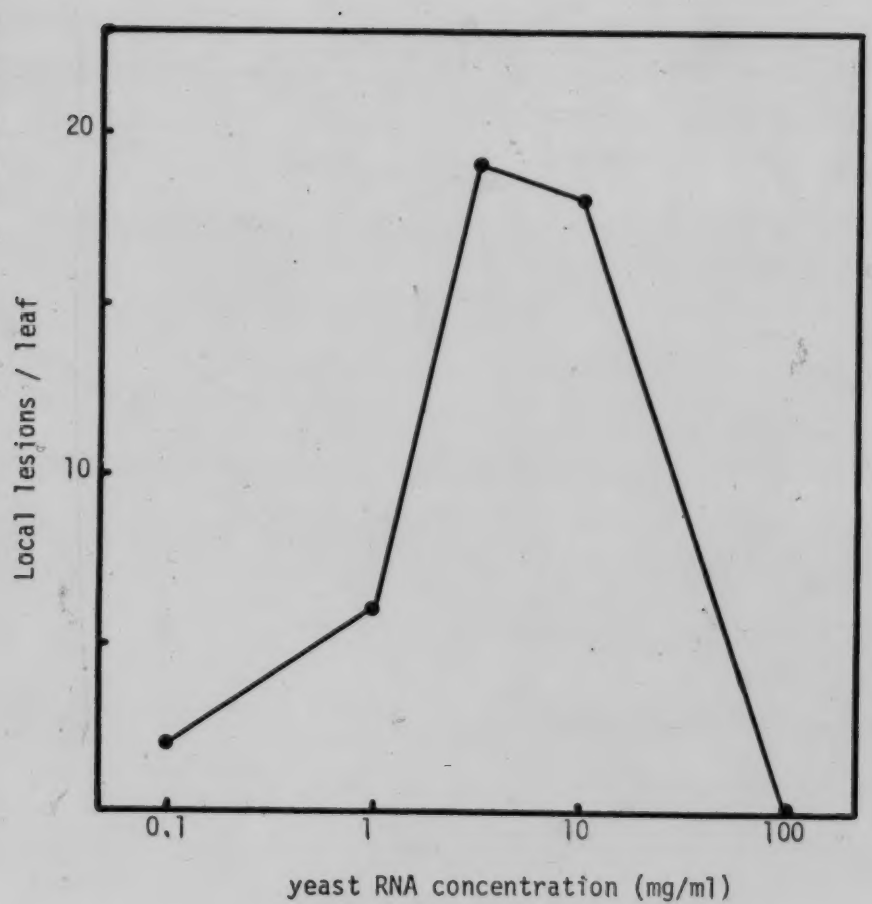
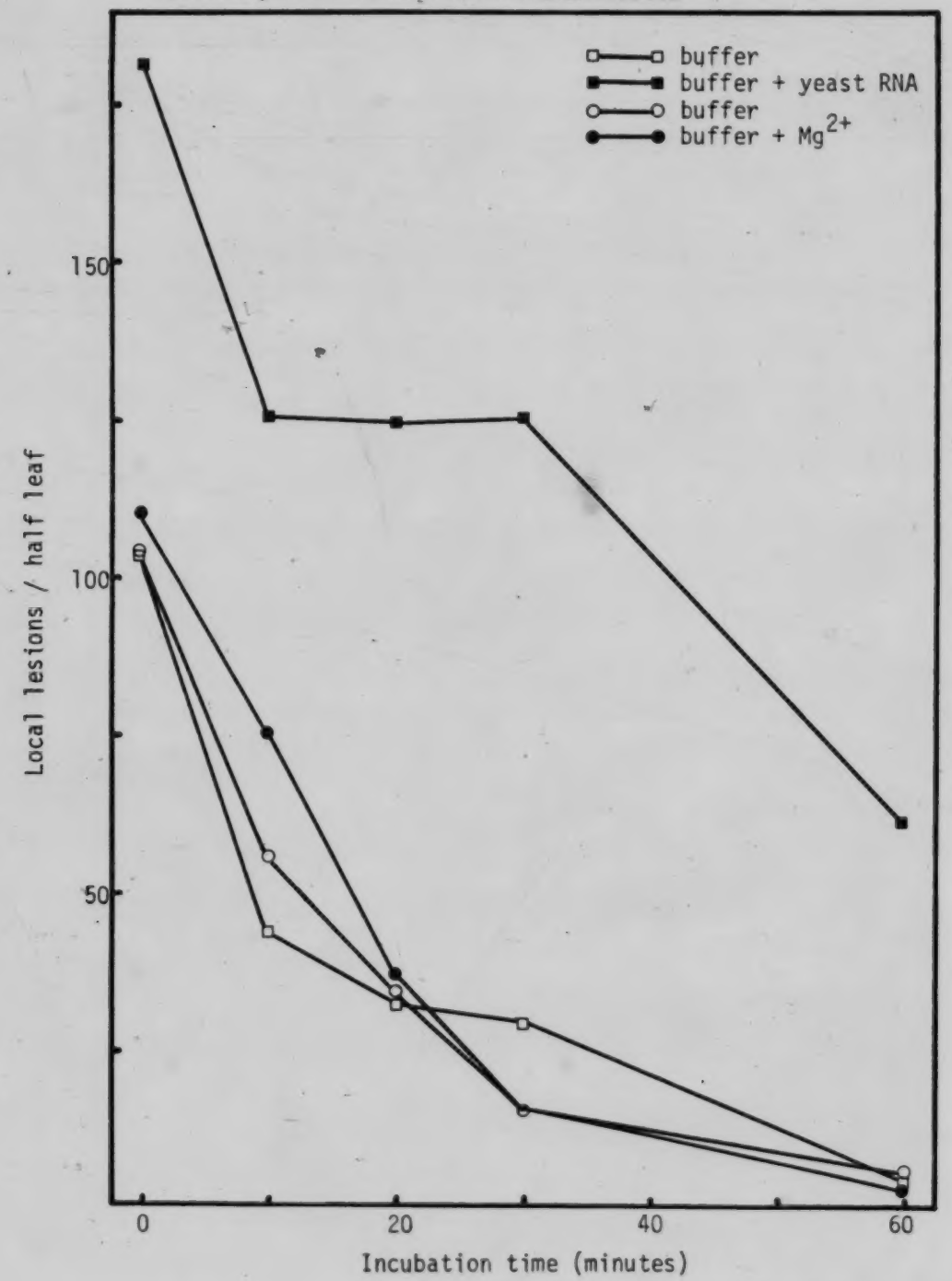


FIGURE 12. Effect of yeast RNA and Mg^{2+} on tobacco stunt agent infectivity in tobacco sap incubated at 20°C and bioassayed on *Chenopodium amaranticolor*



phosphate/PTC buffer containing 10 mg yeast RNA per ml. Samples of these extracts were incubated at 4° and 20°C, and bioassayed for TSA infectivity after known time intervals of incubation. Inoculations were made on half leaves of *C. amaranticolor*. The samples of the same sap extract incubated at 4° and 20°C were inoculated to the two halves of the same leaves.

c) Thermal inactivation point

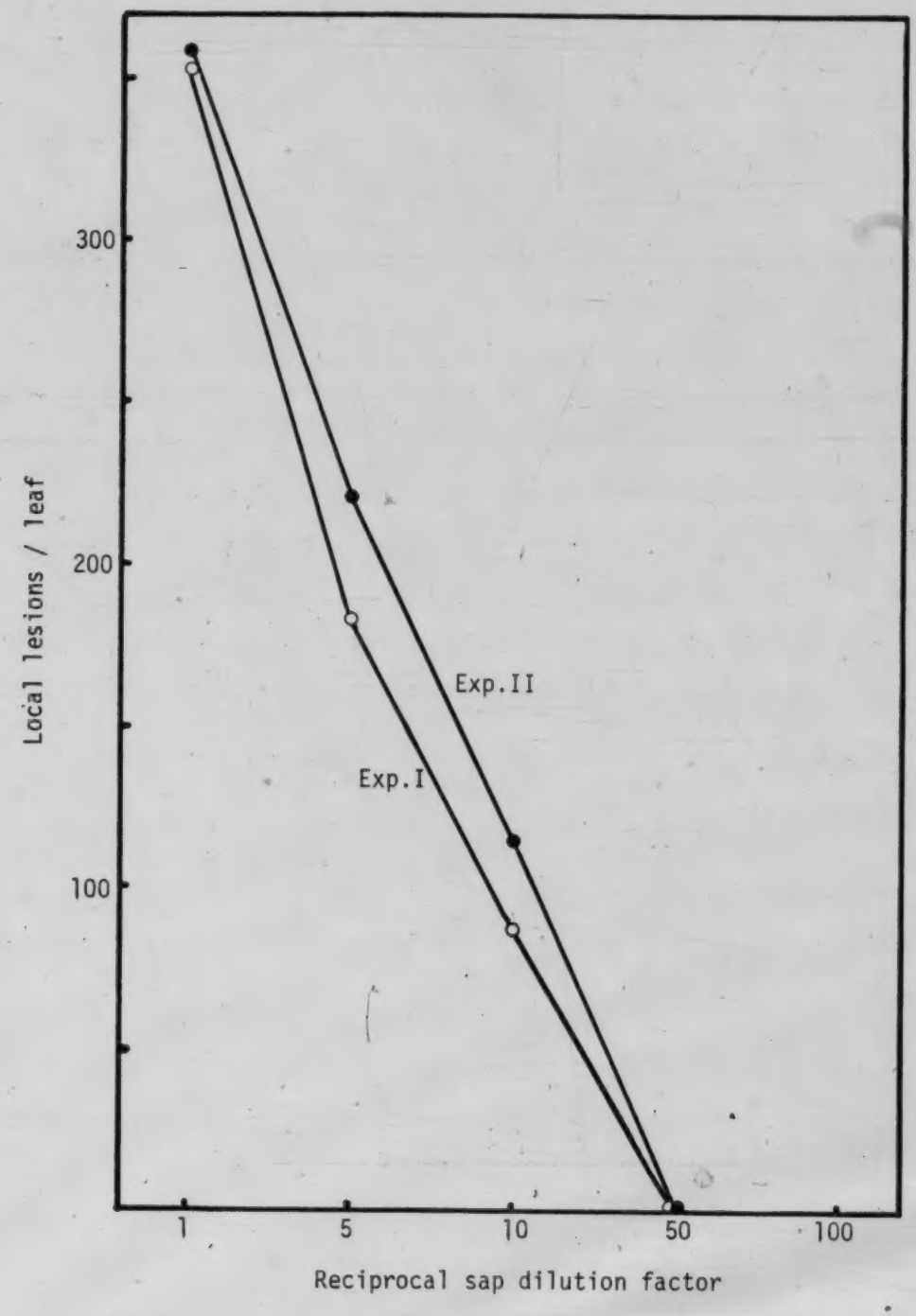
Tobacco sap containing TSA was prepared by homogenising 20 g leaves, showing systemic necrosis, with 20 ml phosphate/PTC buffer pH 7.0 containing 10 mg yeast RNA/ml. After squeezing through cheesecloth, this sap extract was kept on ice. Two ml samples of this sap, in 8 mm diameter tubes, were placed in a water bath at various temperatures ranging from 20° to 50°C at 5° intervals. The sap was maintained at the required temperature for 10 minutes, then cooled to 4°C in an ice bath and bioassayed for TSA infectivity on half leaves of *C. amaranticolor*. The remaining half of each leaf was inoculated with the sap extract which had been maintained at 4°C without any heat treatment. In this way it was possible to compensate for loss of TSA infectivity during the time taken to carry out the series of heat treatments.

2. Results

a) Dilution end point

The dilution end point of TSA infectivity in tobacco sap was found to be between 1:50 and 1:100 (Figure 13). Lesion counts are listed in Table 21 (Appendix).

FIGURE 13. Dilution end point of tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*



b) Longevity

The longevity of TSA infectivity in tobacco sap extracts with or without added yeast RNA was only 2 to 3 hours at 20°C. At 4°C, infectivity was recovered from extracts containing yeast RNA after incubating for 36 hours. However, in the absence of yeast RNA, infectivity was only recovered during the first 24 hours of incubation (Figure 14). Extracts with or without yeast RNA contained very low infectivity when incubated for more than 8 hours at 4°C. Lesion counts are listed in Tables 22 and 23 (Appendix).

c) Thermal inactivation point

Incubation of tobacco sap at 35°C or higher temperatures for 10 minutes completely inactivated TSA infectivity. At 30°C, more than 50% of TSA infectivity was lost. The lesion counts are listed in Table 24 (Appendix).

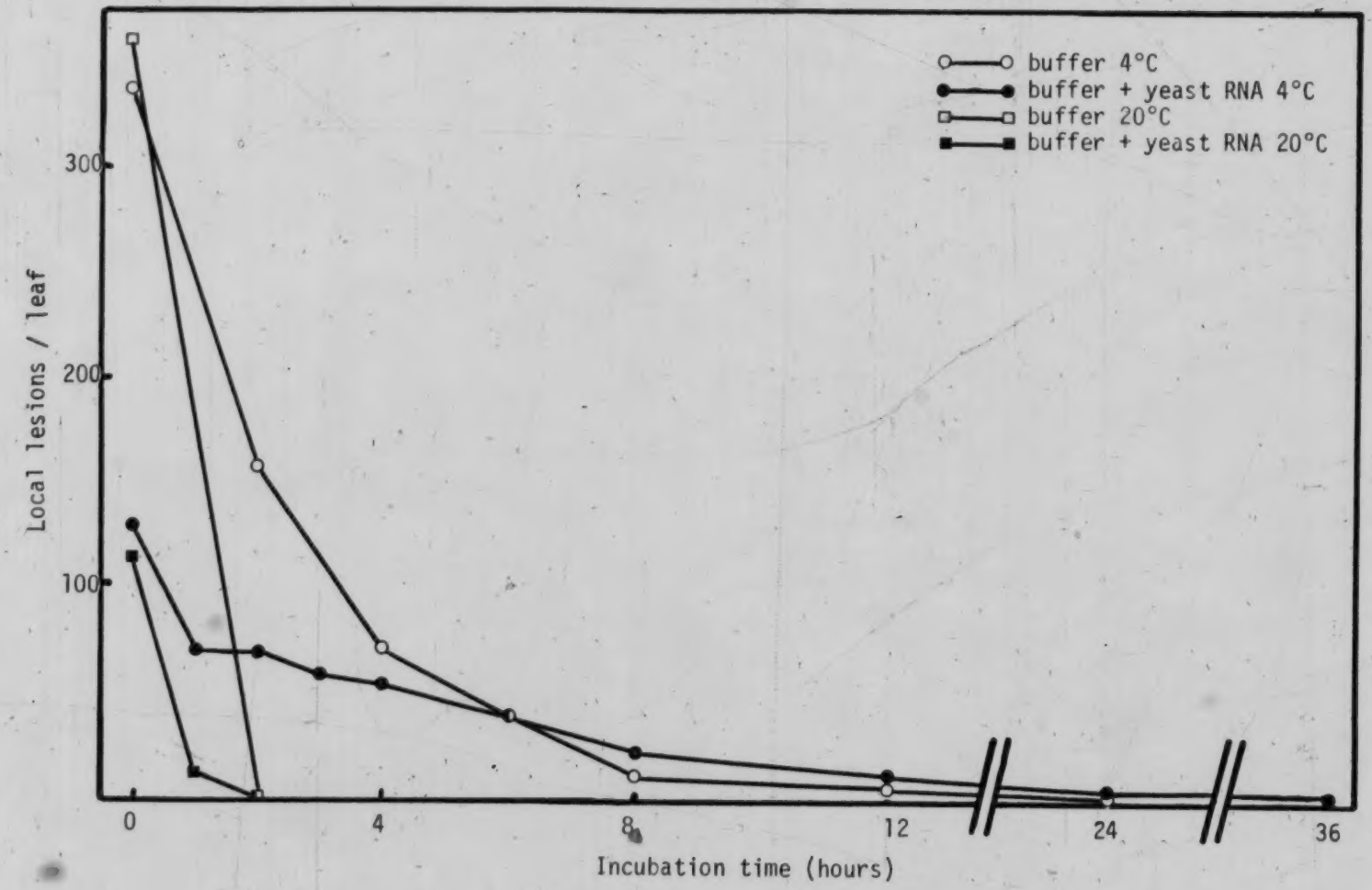
G. ISOLATION OF TSA

1. Materials and methods

a) Extraction of nucleoproteins

Attempts were made to isolate infectious nucleoprotein fractions from tobacco and tomato leaves showing early systemic symptoms of stunt infection. Tomato plants, showing systemic symptoms of vein-clearing and yellowing of young leaves following sap inoculation with TSA (Hiruki, 1975), were used as an alternate source of TSA because infection of the tissues occurs without necrosis. Nucleoprotein extractions were carried out at 4°C using differential centrifugation and polyethylene glycol (PEG) precipitation (Hebert, 1963; McSharry

FIGURE 14. Longevity of tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*



and Benzinger, 1970).

(i) Differential centrifugation

Sap from infected tobacco and tomato leaves was extracted with phosphate buffer pH 7.0, at molarities ranging from 0.01 to 0.5 M, containing 0.001 4-PTC. Tissues were homogenised in a Waring blender for 2 minutes using 2 ml buffer: 1 g leaf tissue. In some experiments 1% ascorbic acid, 1% sodium sulphite or 1% mercaptoethanol were added to the extraction buffer. The tissue homogenate was squeezed through double cheesecloth and bioassayed for TSA infectivity on leaves of *C. amaranticolor* (Chapter III). This expressed crude sap was centrifuged at 13,000 g for 15 minutes (Sorvall RCB-2, rotor SS 34) to remove host material, and the supernatant centrifuged at 105,000 g for 90 minutes (Spinco L-4, rotor 30). Pellets were resuspended in 1 to 2 ml 0.01 M phosphate buffer pH 7.0, and submitted to further cycles of differential centrifugation until the final preparations were relatively free of host material. In some experiments 0.001 M Mg^{2+} was added to the buffer used for resuspending pellets. All pellets and supernatants were bioassayed for TSA infectivity on *C. amaranticolor*. Final preparations were negatively stained with 2% phosphotungstate pH 7.0 for EM examination.

Samples of crude sap extracts were also treated with chloroform, n-butanol or ethanol to aid removal of host material during differential centrifugation. Emulsions of sap plus 2 volumes of organic solvents were centrifuged at 6,500 g for 5 minutes, and the organic layer containing host material was discarded. The supernatants were centrifuged at 81,000 g for 30 minutes, and the resulting pellets resuspended in 1 ml 0.01 M phosphate buffer. These samples were also

bioassayed for infectivity and negatively stained for EM examination. Ultraviolet absorption spectra were recorded for preparations freed of host materials using a Hitachi Perkin-Elmer 139 UV Spectrophotometer.

(ii) Polyethylene glycol precipitation

Infected tobacco and tomato leaves, showing early systemic symptoms, were homogenised with 0.1 M phosphate pH 7.0 containing 0.001 M 4-PTC at a ratio of 2 ml buffer: 1 g tissue. The homogenate was squeezed through cheesecloth and the expressed sap centrifuged at 10,000 g for 10 minutes. This sap extract and subsequent preparations at different stages of extraction were bioassayed for TSA infectivity on *C. amaranticolor*. After removal of some host material by low speed centrifugation, PEG 6000 was added to the supernatant at the rate of 6g/100 ml, and stirred for 15 minutes. This mixture was centrifuged at 5,000 g for 10 minutes and the pellet resuspended in 3 ml 0.01 M phosphate pH 7.0. In preliminary experiments this preparation of TSA was subjected to low speed centrifugation and then precipitated again using PEG. Preparations obtained after one treatment with PEG contained TSA infectivity. Samples of 0.5 ml were layered onto 10 to 40% linear sucrose density gradients prepared in 0.01 M phosphate buffer in 3" x 1" tubes (30 ml capacity). The gradients were centrifuged at 64,000 g for 2 hours (Spinco L-4, rotor SW 25.1), and then fractionated using an Isco Model D Density Gradient Fractionator equipped with a Model UA-2 UV Analyser for recording absorbance at 254 nm. Two ml fractions were collected in separate tubes, and each fraction was bioassayed for TSA infectivity on *C. amaranticolor*.

In a further experiment the fractions containing infectious TSA

were combined, diluted with 4 volumes of 0.01 M phosphate buffer, and then centrifuged at 105,000 g for 90 minutes. The pellet obtained was resuspended in 2 ml phosphate buffer, bioassayed for TSA infectivity and negatively stained for EM examination.

b) Extraction of nucleic acids

Tobacco and tomato leaves showing early systemic symptoms of stunt infection were homogenised for 2 minutes with 1% sodium pyrophosphate or phosphate buffer pH 7.0, molarity ranging from 0.01 to 0.5 M, and water-saturated phenol (Diener and Lawson, 1973). The ratio of 1 g tissue: 1 ml buffer: 1 ml phenol was used. In some experiments bentonite was added to give a concentration of 50 mg/ml. The homogenate was centrifuged at 5,000 g for 10 minutes, and the aqueous layer was extracted again by shaking for 2 minutes with an equal volume of phenol. After centrifugation, traces of phenol remaining in the aqueous layer were removed by ether extraction. Nitrogen gas was then bubbled through the preparation to remove traces of ether, and the nucleic acids were precipitated by the addition of 2 volumes of cold 95% ethanol. This mixture was incubated at -15°C for 30 minutes, and then the precipitate was pelleted by centrifuging at 3,500 g for 10 minutes. The nucleic acids were resuspended in 2 to 3 ml 0.01 M phosphate pH 7.0, and bioassayed for TSA infectivity at dilutions of 1:10 and 1:100 on leaves of *C. amaranticolor*. Preparations were also diluted for determining the UV absorption spectra.

Nucleic acid samples were submitted to polyacrylamide gel electrophoresis using 2.6% gels in 0.6 x 10 cm perspex tubes. Gels

were prepared using recrystallised acrylamide and bisacrylamide (Adesnik, 1971; Loening, 1967, 1969). Nucleic acid preparations were diluted 1:10 and 1:20 with electrophoresis buffer and 0.3 ml samples were layered on each gel. The gels were subjected to electrophoresis for 2 hours at 3 mAmp per tube. At the completion of the run, gels were stained for 1 minute with 0.05% toluidene blue in 0.01 M sodium acetate pH 5.5. Destaining was carried out for 1 hour in 0.05 M sodium acetate pH 5.5 diluted 1:10. To test experimental technique, purified brome mosaic virus (BMV) RNA was layered onto similar gels and subjected to electrophoresis.

c) Negative staining of TSA preparations for TEM

Preparations of nucleoproteins obtained from infected tobacco and tomato tissues as outlined in a) were applied to Formvar coated TEM grids, and negatively stained with 2% aqueous phosphotungstic acid (PTA) adjusted to pH 7.0 with 0.1 N sodium hydroxide.

Dip preparations were also made for TEM examination from leaves, stems and petioles of infected tobacco and tomato showing symptoms at different stages of development. The cut surfaces of tissues were dipped into drops of glass distilled water on formvar-coated grids. Excess water was removed and the grids negatively stained with PTA. After air-drying they were examined with the TEM.

2. Results

a) Extraction of nucleoproteins

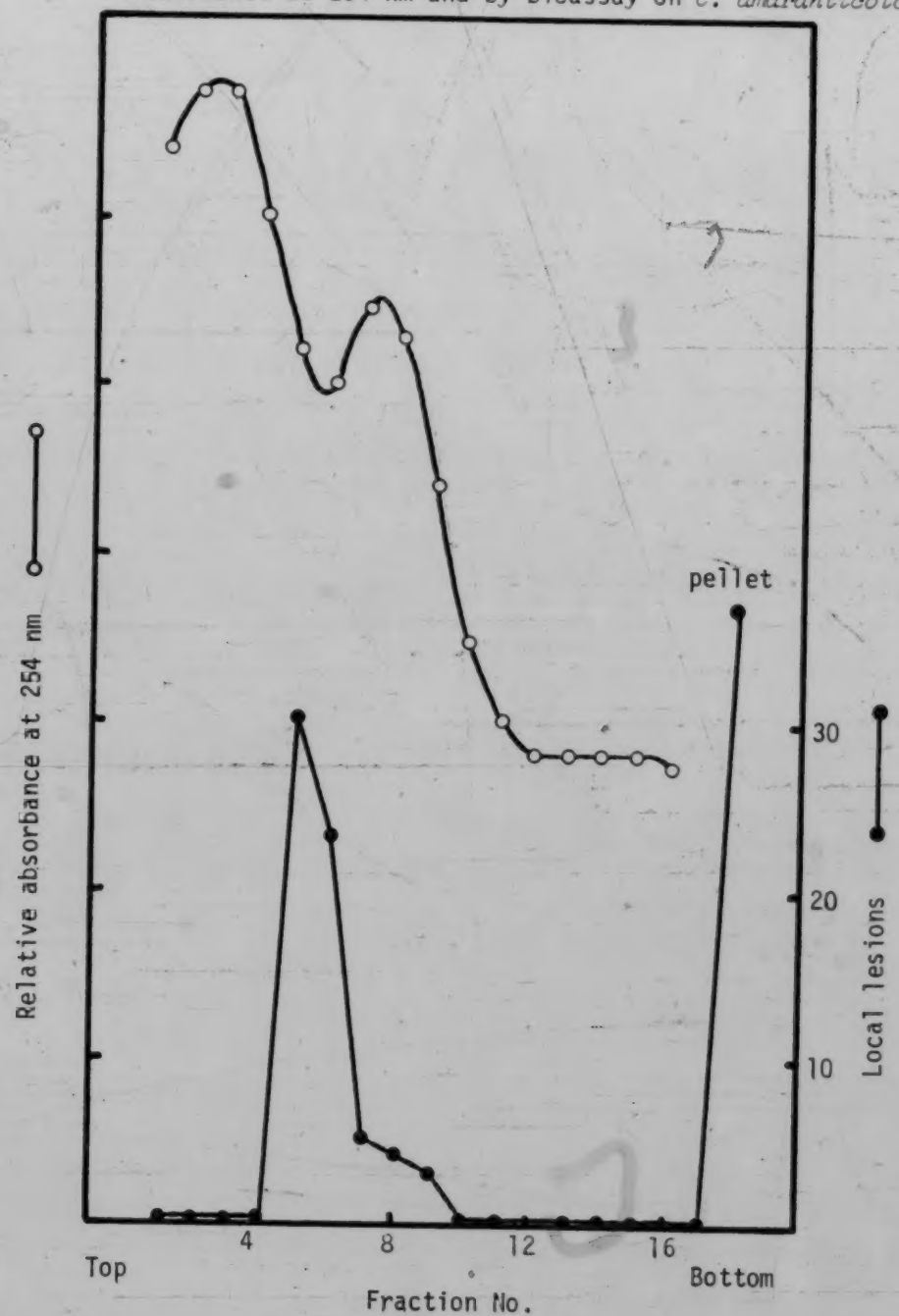
An infectious preparation of nucleoprotein was obtained from tomato leaves following clarification of the sap with chloroform and differential centrifugation. The crude sap extracted in 0.1 M phosphate

pH 7.0 containing 0.001 M 4-PTC did not contain any TSA infectivity. However the clarified centrifuged preparation was infectious on leaves of *C. amaranticolor* (mean count of 43 lesions/leaf). No infectivity was obtained on leaves of Red Kidney bean. No distinct virus-like particles were observed in negatively stained samples from partially purified preparations of sap from diseased and healthy tomato plants.

No infectious preparations were obtained from tobacco leaves following differential centrifugation. The addition of anti-oxidants to the extraction buffer and adjustment of the buffer molarity had no effect on stabilising TSA which gave low infectivity in the crude sap extracts. Small particles, 8-9 nm in diameter (Plate 30,A), were observed in non-infectious preparations obtained after 2 cycles of differential centrifugation.

After one treatment with PEG, the infectious TSA present in sap prepared from tobacco and tomato leaves was concentrated more than four-fold, as determined by local lesion counts. However, this infectivity was completely lost after a second PEG treatment, and green host material was still present in the samples. Virus-like particles, 15 to 35 nm in diameter, were observed in negatively stained preparations (Plate 30, B & C). A wide range of particle sizes was observed. Some particles were as large as 45 to 50 nm in diameter, and others appeared to be surrounded by an envelope. Following SDG centrifugation, TSA infectivity was recovered from fractions 5 to 9 (from the top of the tube), and from the green pellet of host material (Figure 15). The highest infectivity was obtained from fractions 5 and 6 and the resuspended pellet. Absorbance

FIGURE 15. Fractions of preparation from tobacco stunt agent - infected tobacco obtained by sucrose density gradient following PEG precipitation, and monitored by relative absorbance at 254 nm and by bioassay on *C. amaranticolor*



at 254 nm showed a peak around fractions 7 and 8. The infectivity obtained after SDG centrifugation was lower than in the PEG treated sap. Likewise, infectivity decreased further following ultracentrifugation of the combined infectious SDG fractions.

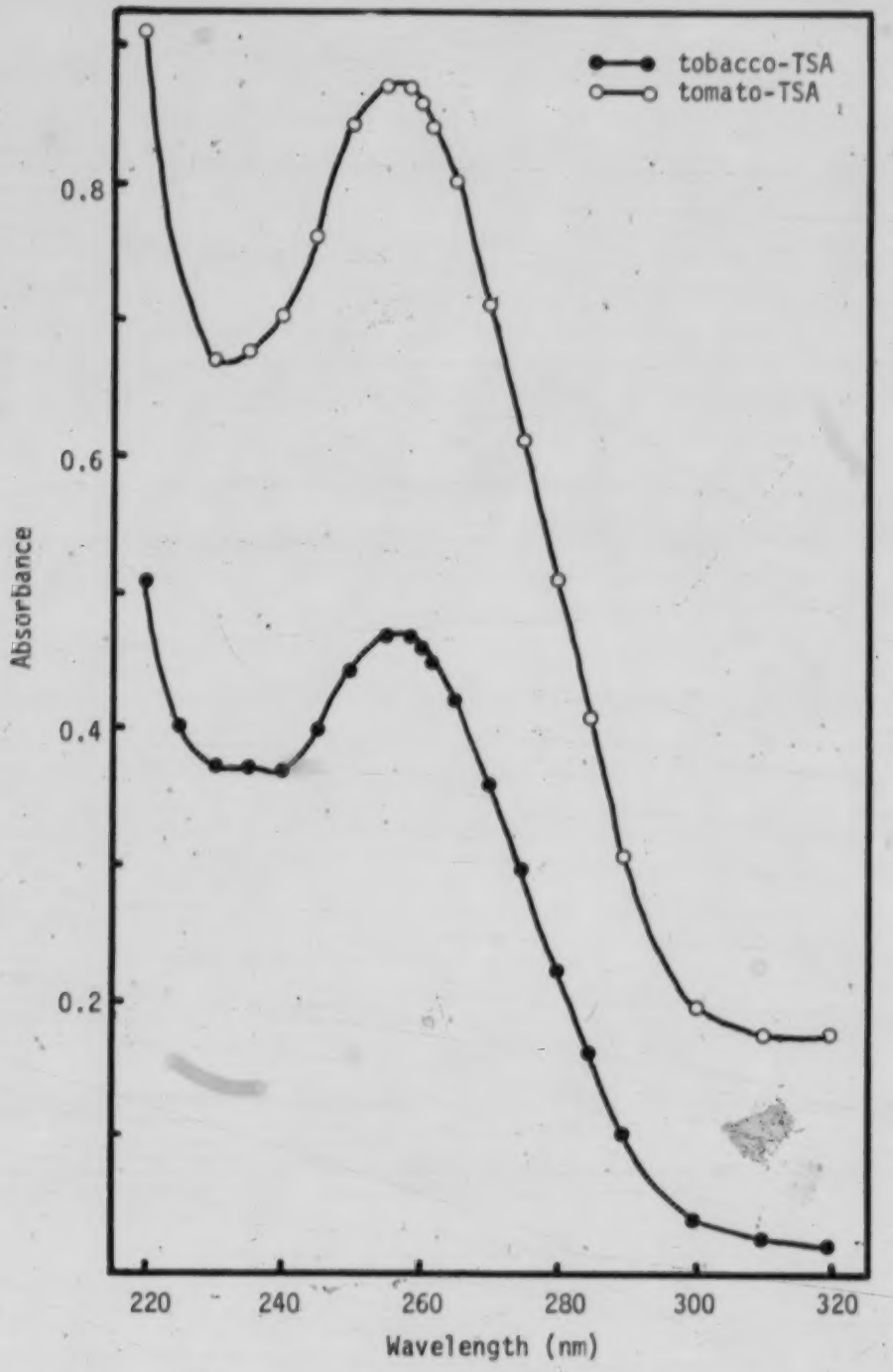
b) Extraction of nucleic acids

All preparations of nucleic acids from infected tobacco and tomato tissues were off-white in colour, non-infectious, and gave UV absorption spectra showing considerable protein contamination (Figure 16). Buffer molarity and the addition of bentonite during extraction had no effect on the recovery of infectious nucleic acids. After electrophoresis, no extra RNA bands were located in gels containing the nucleic acids isolated from stunt infected tobacco and tomato tissues. Two bands of host RNA were present in both healthy and diseased preparations. Purified BMV RNA, after electrophoresis, showed three bands, representing RNA 1 + RNA 2 (1.09×10^6 d. and 0.99×10^6 d. respectively), RNA 3 (0.75×10^6 d.), and RNA 4 (0.28×10^6 d.) (Lane and Kaesberg, 1971). The band for RNA 4 was faint in contrast with the bands for RNA 1 + RNA 2 and RNA 3.

c) Negative staining of TSA preparations for TEM

Virus-like particles 8 to 9 nm and 15 to 50 nm in diameter were observed in partially purified nucleoprotein preparations obtained from stunt infected tobacco and tomato. However, these preparations did not contain TSA infectivity. Also no infectivity was obtained on Red Kidney bean. No virus-like particles were observed in negatively stained dip preparations of tissues of infected tobacco and tomato. Also no particles were detected in dip preparations made

FIGURE 16. UV absorption spectra of phenol-extracted preparations from tobacco and tomato leaves infected with tobacco stunt agent



from TSA local lesions on leaves of *C. amaranticolor*.

H. DISCUSSION

Although attempts to characterise and visualise TSA were not successful, some important information about the nature of this agent was obtained. Tobacco stunt agent was extremely unstable in sap preparations and readily inactivated during purification procedures. Due to differences in infected tobacco and in sensitivity of *C. amaranticolor* leaves, TSA infectivity varied within and between experiments.

The stage of symptom development influenced the recovery of TSA infectivity from infected tobacco. Highest infectivity was obtained when early systemic symptoms were visible. Following the development of severe necrosis and stunting, TSA infectivity was reduced. Temporary remission of stunt symptoms occurred in infected plants grown at 25°C, whereas at 33°C symptoms were lost permanently. This heat therapy (Hidaka and Hiruki, 1958), may have been due to the effect of high temperatures directly on TSA or on the interaction between TSA and the tobacco host. Temperatures also had an effect on the number of local lesions which developed on leaves of *C. amaranticolor* following sap inoculation with TSA. Plants incubated at 33°C did not develop any visible lesions. The thermal inactivation point of TSA in tobacco sap was found to be 10 minutes at 35°C. This would suggest that the effect of temperature was on TSA and not the interaction with the host. However, this is not certain because the thermal inactivation point for TSA in sap has also been reported to be between 75 and 80°C (Hiruki, 1975). This variation in results may be due to differences in

the source of TSA or experimental conditions. In the present study TSA was maintained on tobacco by serial mechanical transmission.

Treatment of infected tobacco at different stages of symptom development with antibiotics did not produce any remission of symptoms. Addition of penicillin and chloramphenicol to TSA in tobacco sap did not significantly reduce infectivity. The reduction of infectivity associated with the addition of tetracycline was only significant in this experiment at the 5% level. In other tests the reduction of infectivity was not significant. This illustrates some of the variation obtained in bioassaying TSA infectivity. Buffer containing antibiotics was maintained at pH 7.0 to avoid any effects of pH on TSA infectivity. From the results of *in vivo* and *in vitro* treatments of TSA with antibiotics it is suggested that stunt disease in tobacco is not caused by a bacterium, rickettsia or Mollicutes-like organism.

Infectivity of TSA in tobacco sap was reduced by low pH and by high molarity of phosphate buffer. Sodium sulphite had no effect on infectivity, and the reduction caused by the addition of ascorbic acid was probably due to an effect of low pH. Addition of the chelating agent, 4-PTC, significantly increased the level of TSA infectivity recovered from tobacco sap. The activity of host polyphenol oxidases can be reduced by Cu^{2+}-chelating inhibitors (Hampton and Fulton, 1961), and since the activity of these enzymes was reported to be higher in tobacco infected with certain plant viruses than in healthy plants (Martin, 1958), 4-PTC may stabilise TSA infectivity by the chelation of Cu^{2+} and subsequent inhibition of host polyphenol oxidases. The unstable nature of TSA in tobacco sap was shown by the longevity of 2 hours at 20°C. Even at 4°C, infectivity was only

obtained for 24 hours. The dilution end point of between 1:10 and 1:100 suggests that tobacco tissues do not contain high concentrations of TSA, and also that the infectious agent is unstable in sap.

The complete inactivation of TSA infectivity in tobacco sap by the addition of RNase at concentrations higher than 0.1 µg/ml suggests that TSA contains RNA. In contrast, DNase had no significant effect at the highest tested concentration of 100 µg/ml. The significant reduction of infectivity associated with the addition of 500 µg protease/ml was not due to a pH effect, since the pH of the extraction buffer remained at 7.0. It is suggested that TSA contains a protein coat which is degraded by protease and leaves the infectious RNA vulnerable to inactivation by host nucleases. A loose arrangement or packing of the subunits in such a protein coat would explain the unstable nature of TSA in tobacco sap, and the rapid inactivation by added RNase. The protein coat may not be loosely packed in all particles since inactivation by added RNase, over an incubation period of 1 hour, was rapid during the first 10 minutes but then levelled off. Also protease at a concentration of 2 mg/ml did not completely inactivate TSA.

The reduction of infectivity associated with the addition of Mg-bentonite and bentonite to tobacco sap also suggests that TSA may contain a protein component, since nucleoproteins can be adsorbed by clays such as bentonite. Likewise the precipitation and concentration of infectious TSA by PEG, and fractionation by SDG centrifugation indicate that TSA may be a nucleoprotein. The variation in sizes of particles observed in non-infectious preparations following PEG precipitation may be due to random aggregation of the protein sub-units,

or represent stages in the disruption of the protein coat. If the latter theory is correct, then the infectious particles of TSA are large in comparison with many plant viruses.

Sensitivity of TSA to phenol extraction suggests that it does not exist as a free nucleic acid. This is also supported by sensitivity to high temperatures, and the lack of stabilisation of the infectious agent by bentonite and by high molarity phosphate buffer. The addition of yeast RNA partially protected TSA from inactivation by providing the host nucleases with an alternate substrate. The reduction of infectivity associated with yeast RNA concentrations higher than 10 mg/ml was probably due to a molarity effect, because the buffer had to be adjusted to pH 7.0 with sodium hydroxide.

The results obtained in the present study suggest that TSA is an infectious RNA which is protected by a very loose protein coat. In contrast with conventional plant viruses and viroids, this disease agent is extremely unstable in extracted sap. Although the presence of a protein coat is suggested, it is still possible that TSA is an infectious RNA associated with a protein component of the host cell.

CHAPTER VI

MORPHOLOGY AND ULTRASTRUCTURE OF *OLPIDIUM* AS RELATED TO ITS ROLE AS VECTOR OF TOBACCO STUNT AGENT

A. INTRODUCTION

The ultrastructure of a lettuce isolate of *Olpidium brassicae* has been investigated as regards its transmission of tobacco necrosis virus (TNV) (Temmink and Campbell, 1968, 1969a, 1969b; Temmink, 1971). Prepenetration and penetration stages of a cabbage isolate of *O. brassicae* have also been reported (Lesemann and Fuchs 1970a, 1970b). The present study involving a tobacco isolate of *Olpidium* carrying TSA was carried out for the following reasons;

1. to compare the life-cycle, morphology and ultrastructure of this tobacco isolate with reports of other isolates, and to determine whether TSA has any effect upon its vector.
2. to attempt visualisation of TSA in different stages of its vector.
3. to determine the stages of *Olpidium* concerned with acquisition and transmission of TSA.
4. to investigate stages in the life-cycle of *Olpidium* not previously reported in detail.

The association between *Olpidium* and TSA has been shown to be highly stable and persistent (Hiruki, 1965, 1968, 1972). Acquisition of TSA by *Olpidium* only occurs *in vivo* (Chapter IV; Hiruki, 1965), and it has been proposed that the agent is present inside the vector. Soejima and Hidaka (1969) reported virus-like

particles in cells in a pellet of *Olpidium*/TSA zoospores, and suggested that the agent multiplies in its vector. However, the identity of these particles as the infectious agent of tobacco stunt was not demonstrated. Attempts have been made to verify this report, and to determine whether TSA has any effect on its vector because of its internal and persistent association. It has been proposed that transmission of TNV occurs within 24 hours after penetration of the host cell by the fungal thallus (Temmink and Campbell, 1969b; Temmink, 1971), during which time the fungal cytoplasm and host cytoplasm are separated by only a single membrane. In this study the isolate of *Olpidium*/TSA was examined to determine the stage or stages in the life-cycle of the fungus during which transmission and *in vivo* acquisition of the internally associated TSA could occur.

Two stages in the life-cycle of *Olpidium* have not been reported in detail, i.e. the retraction of the flagellum by zoospores, and the morphology and ultrastructure of resting sporangia. Observations on the retraction of the flagellum by some fungal zoospores have been made with the light microscope using time-lapse photography (Aist and Williams, 1971; Holloway and Heath, 1974; Koch, 1968). Different retraction methods for chytrid zoospores were proposed (Koch, 1968), and the genus *Olpidium* was grouped according to the 'lash-around' or 'wrap-around' method. Membrane fusion was suggested to occur during retraction of the flagellum, but the fate of the flagellum membrane was not demonstrated. The method by which the flagellum is retracted may be significant with respect to the transmission of any virus or virus-like agents associated with the flagellum (Temmink and Campbell, 1969b; Temmink, 1971).

The small zoospores of *Olpidium* make observations of prepenetration stages with the light microscope very difficult. However, use of the SEM allows observation of these stages in much greater detail.

To date, observations of isolates of *Olpidium* resting sporangia have only been made at the light microscope level (Bensaude, 1923; Sampson, 1939; Sahtiyanci, 1962). No ultrastructural details have been reported. Infectivity of TSA has been obtained from air-dried resting sporangia retained for at least seven years after TSA acquisition (Hiruki, 1972), and the stunt agent in resting sporangia survives acid, heat and UV irradiation treatments as long as the fungus remains viable. Because of this persistence of TSA in resting sporangia, the morphology and ultrastructure of these stages were included in this study.

The scanning electron microscope is now frequently used for examination of fungi. Observations of specimens can be made over a wide range of magnifications, duplicating and supplementing information obtained from the light microscope, and complementing observations of ultrastructure made with the TEM. This technique has been applied to studies of the microflora on plant roots (Campbell and Rovira, 1973; Dart, 1971; Locci, 1969a; Rovira and Campbell, 1974), and the invasion of leaf tissues by zoospores (Locci, 1969b; Royle and Thomas, 1971; Royle and Thomas, 1973). However, this technique has been used very little for studying the stages of zoospore infection on plant roots (D'Ambra and Locci, 1971). Recently SEM has been used to determine the distribution and relationships of fungi within plant tissues (Jones *et al.*, 1974; Kinden and Brown, 1975; Murphy *et al.*, 1974; Welch and

Martin, 1973), however, only one report has been published involving fungi in plant root cells (Lutz and Sjolund, 1973).

B. LIFE-CYCLE OF *OLPIDIUM*

1. Materials and Methods

Roots of 6 days old tobacco seedlings, grown at 25°C in sterilised quartz sand, were washed free of sand particles, and then incubated in concentrated zoospore suspensions of *Olpidium* and *Olpidium*/TSA (Chapter III) for 15 minutes. After this pulse inoculation, the roots were washed twice in distilled water, and incubated in a half-strength Hoagland's solution in an incubator at 17°C with 16 hours light period, and light intensity of 5,000-10,000 lux. Roots inoculated with each *Olpidium* isolate were sampled at known time intervals, mounted in water on slides and examined with a light microscope (Chapter III). Free zoospores were observed live in distilled water, and also after fixation with vapour from a 2% solution of osmium tetroxide. Fixation was achieved by placing a drop of zoospore suspension on a glass slide and inverting it over the osmium tetroxide solution for 5 minutes.

2. Results

Stages in the life-cycle of the tobacco isolate of *Olpidium* are illustrated in Plate 4, Free zoospores were observed with a round body and a single flagellum. The nucleus and some granules were visible in the zoospore body (Plate 4,A). During the pulse inoculation period, zoospores attached to the tobacco roots mainly in the zone of cell elongation. However, some zoospores also attached to root hairs. Two hours or more after attachment,

penetration of the host cell wall by the zoospore cytoplasm occurred (Plate 4,B). After incubating for 48 hours, characteristic zooporangia were visible in the root epidermal cells (Plate 4,D). After a further 48 hours mature zoosporangia with exit tubes were present, and release of zoospores occurred (Plate 4,E). Resting sporangia were also observed with characteristic, thick, undulating walls (Plate 4,C). Following pulse inoculation of tobacco roots with *Olpidium* and *Olpidium*/TSA zoospores, mature zoosporangia developed and released the subsequent generation of zoospores in 3 to 4 days. No differences between the life-cycles of the two isolates of *Olpidium* were observed.

C. FREE ZOOSPORES

1. Materials and methods

Concentrated zoospore suspensions (Chapter III) of *Olpidium* and *Olpidium*/TSA were prepared for SEM examination using the following fixation and dehydration schedules:

a. Zoospores on Millipore filter. Samples of the zoospore suspensions were placed on small pieces of Millipore filter (50 nm pore size), and treated in one of the following ways. (i) The Millipore was frozen in liquid Freon 13 and then transferred to liquid nitrogen prior to drying in an Edwards Speedivac II. (ii) The Millipore was fixed in osmium vapour and then gently washed in distilled water, followed by dehydration through a graded ethanol series, and critical point dried from absolute ethanol (DeBault, 1973).

b. Zoospores in Nuclepore 'bags'. Samples of the zoospore suspensions

were put into small tubes and treated in one of the following ways. (i) Fixation for 1 hour at room temperature after adding an equal volume of 1% potassium permanganate in distilled water. After fixation the zoospores were pelleted by centrifuging at 3,500 g for 5 minutes (Sorvall RCB-2, SS 34 rotor), and resuspended in a few drops of distilled water. Samples were put into Nuclepore 'bags' (Atwood *et al.*, 1975) made by lining porous teflon capsules with Nuclepore membrane. (5 μm pore size), and dehydrated through a graded ethanol series followed by critical point drying from absolute ethanol. (ii) Fixation for 30 minutes after adding an equal volume of 2% osmium tetroxide in distilled water. Following fixation the zoospores were washed, dehydrated and dried as in (i). (iii) Prefixation for 1 hour after adding an equal volume of 0.01 M phosphate buffer pH 7.0 containing 2% glutaraldehyde plus 2% formaldehyde. After fixation the zoospores were pelleted by centrifuging at 3,500 g for 5 minutes, resuspended in distilled water, and postfixed for 30 minutes by adding an equal volume of 2% osmium tetroxide in distilled water. The zoospores were then washed, dehydrated and dried as in (i).

The same concentrated zoospore suspensions were also processed for sectioning for transmission electron microscopy. Zoospores were fixed by adding an equal volume of 4% glutaraldehyde plus 4% formaldehyde in 0.01 M phosphate pH 7.0 to the suspensions, and leaving at room temperature for 2 hours. The fixed zoospores were pelleted by centrifugation, and washed twice for 30 minutes in distilled water. A few drops of 2% water agar at 45°C were added to the zoospore pellets, and the agar allowed to gel. The agar containing zoospore

pellets was cut into small pieces and postfixed in 2% osmium tetroxide in distilled water for 2 hours at room temperature. The samples were then washed, dehydrated through a graded ethanol series, embedded in Araldite, sectioned and stained for observation with the TEM (Chapter III).

Free zoospores were also observed with the TEM after osmium vapour fixation and negative staining. The zoospore suspensions were washed twice in distilled water by centrifugation. Drops of these suspensions were placed on Formvar coated electron microscope grids on dental wax in petri plates. Drops of 4% aqueous osmium tetroxide were placed on the wax, and the zoospores fixed in osmium vapour for 30 minutes. Half of the grids were then air-dried, and the remainder were negative-stained with 2% aqueous phosphotungstate pH 7.0. Grids were then examined with the TEM (Chapter III).

2. Results

In the SEM, each free zoospore had a round body, 2-3 μm in diameter, and a single whiplash flagellum 14-17 μm in length (Plate 7,A). The body had a ruffled outline and was never observed to be perfectly smooth. In the region where the flagellum joins the body a ring-like swelling was observed (Plate 7,B&C). The best preservation of free zoospores was achieved using either osmium vapour with zoospores on Millipore filter (Plate 7,A&B) or aqueous osmium tetroxide with zoospores in the Nucleopore 'bags' (Plate 7,C), followed by critical point drying. The critical point drying was carried out directly from ethanol since amyl acetate dissolves Millipore and softens Nuclepore to a slight extent. The Nuclepore 'bags' were extremely convenient for handling zoospore samples with

the minimum amount of physical disturbance. Prefixation with aldehydes followed by postfixation in osmium tetroxide resulted in an irregular outline of the zoospore body when compared with osmium fixation alone. Collapse of the body occurred when permanganate was used for fixation. Freeze-drying with or without fixation produced considerable collapse and damage to the zoospore body. In comparison, critical point drying gave excellent preservation following osmium fixation.

In the TEM zoospores fixed in osmium vapour were also observed with a round body and a single whiplash flagellum (Plate 14,A). The membrane surrounding the body and flagellum of each zoospore was visible, but no virus-like particles attached to the membrane were observed in negatively stained samples. The body was electron-dense with some osmiophilic bodies visible.

Zoospores sectioned for TEM possessed a plasmamembrane continuous around the body and flagellum (Plate 14,B&C). In some sections the spatial relationships between the flagellum, kinetosome, rhizoplast and nucleus were observed. In longitudinal sections of the attachment of the flagellum to the body (Plate 14,C), the two central fibrils of the flagellum terminated at the kinetosome. Transverse sections of the flagellum showed the typical 9+2 arrangement of the fibrils (Plate 14,D). In the region of the kinetosome, where the flagellum joins the body, a slight swelling was observed (Plate 14,B).

No differences in the structure of *Olpidium* and *Olpidium*/TSA zoospores were observed. No virus or virus-like particles were visible inside or external to the body and flagellum of *Olpidium*/TSA zoospores.

D. ZOOSPORES ATTACHED TO TOBACCO ROOTS

1. Materials and methods

Roots of healthy tobacco seedlings germinated in moist sand at 25°C for 6 to 10 days were incubated in concentrated zoospore suspensions (Chapter III) of *Olpidium* and *Olpidium*/TSA, and sampled at known time intervals up to 20 minutes of incubation. At the end of this time the remaining roots were washed twice with distilled water and incubated under the same conditions, as outlined in section B, for a further 12 hours. Roots sampled during both incubation periods were examined in the light microscope, fluorescence microscope, SEM and sectioned for TEM.

Roots for light and fluorescence microscopy were mounted fresh in aniline blue stain preparation (Chapter III).

Roots for SEM examination were fixed and dried in the following ways. (i) Roots were frozen in Freon 13 and then transferred to liquid nitrogen for freeze-drying (see Section C). (ii) Fixation for 1 hour in 0.01 M phosphate buffer pH 7.0 containing 2% glutaraldehyde plus 2% formaldehyde, washed with distilled water and then freeze-dried as in (i). (iii) Prefixation in glutaraldehyde and formaldehyde as in (ii), washed in distilled water, postfixed in 2% aqueous osmium tetroxide for 30 minutes, washed again and then freeze-dried as in (i). (iv) Prefixation and postfixation as in (iii). Fixed roots were washed in distilled water and transferred to porous Teflon capsules, prior to dehydration through an ethanol series, and critical point dried from amyl acetate (Anderson, 1951). (v) Fixation in 2% aqueous osmium tetroxide for 30 minutes, washed and dehydrated as in (iv). (vi) Fixation in 1% aqueous potassium permanganate for

1 hour, washed and dehydrated as in (iv).

Some roots were also freeze-fractured after fixation for 4 to 6 hours at room temperature in 0.1 M phosphate buffer pH 7.0 containing 2% glutaraldehyde plus 2% formaldehyde, and postfixation in 2% aqueous osmium tetroxide for 2 to 4 hours. Fixed roots were frozen rapidly in Freon 13 and fractured with a pre-cooled scalpel. The fractured roots were transferred into liquid nitrogen, freeze-dried and examined with the SEM (Chapter III).

Roots sampled for observation with TEM were fixed, dehydrated, embedded and sectioned as outlined in Chapter III.

2. Results

Light microscopy and SEM observations showed that zoospores attached to the tobacco roots mainly in the zone of cell elongation (Plate 5,A&C; Plate 9,B&C), which is situated up to 2 mm behind the root cap. When a high concentration of zoospores ($>10^7$ zoospores/ml) was used as inoculum, they were also seen attached to root hairs (Plate 9,D). Many zoospores were attached to roots sampled between 5 and 10 minutes after incubation. Zoospores were observed with the flagellum still present and probably not retracting, since the ring-like swelling was still visible (Plate 7,D&E). The flagella of some zoospores were coiled or looped (Plate 8,A&B). In some cases the flagellum was observed wrapped around the body and merging of the flagellum and body plasmamembranes was apparent (Plate 8,C&D). Other zoospores were observed with the flagellum wrapped around the body and only the whiplash remaining free (Plate 8,E). The body of each zoospore undergoing retraction of the flagellum appeared

flattened with numerous surface irregularities showing a possible ridge formation. However, roots sampled after 20 minutes of incubation showed round encysted zoospores with a relatively smooth outline and with the flagellum completely retracted (Plate 8,F).

For SEM examinations the preservation of the zoospore body when attached to tobacco roots varied according to the fixation procedure used. An irregular outline of the body was frequently observed (Plate 7,E; Plate 8,A) even though later stages showed good preservation following the same fixation procedure. Fixation in osmium alone (Plate 7,D) or prefixation in aldehydes followed by osmium (Plate 7,E) were the best procedures for preservation of the zoospore body. Critical point drying gave better results than freeze-drying with or without the use of prefixation. Permanganate as a fixative before critical point drying resulted in considerable collapse of the zoospore body.

Roots sampled 10 minutes after incubation and sectioned for TEM showed attached zoospores with an irregular discoid outline and limited by only the body plasmamembrane (Plate 15,A,C&D). Plate 15,A shows an oblique section through an attached zoospore which is retracting the flagellum. The body is flattened and surrounded by only the plasmamembrane. The 9+2 fibrils of the flagellum (axoneme) are surrounded by cytoplasm (Plate 15,A&B), indicating that retraction is in progress. The flagellum plasmamembrane is absent. After retraction of the flagellum the axonemal fibrils without the surrounding membrane were seen in oblique and transverse sections within the body cytoplasm (Plate 15,C&D). A cyst wall was deposited exterior to the body plasmamembrane soon after retraction

of the flagellum (Plate 16,A&B), and the zoospore body then assumed a comparatively round outline. One hour after inoculation the cyst wall around the zoospore body was quite distinct (Plate 17,A&B).

Light and fluorescence microscopy of roots incubated for two hours or more revealed fluorescence due to callose substances in areas adjacent to encysted zoospores (Plate 5,A&B, C&D). Fluorescence was associated with the thickening of the host cell wall in response to penetration by zoospores (Plate 5,E&F). Roots incubated for 2 hours and freeze-fractured for SEM examination showed encysted zoospores with adjacent thickening of the host cell wall (Plate 9,F; Plate 10,A). Some fractured cysts contained a large vacuole distal to the site of attachment to the host cell wall (Plate 9,E). Fractured roots at 3 and 4 hours after inoculation contained cysts and penetrating thalli (Plate 10,B&C). Cysts at 24 hours after inoculation were often collapsed and adjacent cell wall thickenings were still visible (Plate 10,D). One empty cyst on the root surface was damaged during freeze-fracturing, and the hole in the cell wall made by the penetrating thallus was revealed (Plate 10,E).

Examination of sections of encysted zoospores with the TEM showed the presence of a definite cyst wall after 1 hour of incubation. Axonemal fibrils seen in transverse section were irregular in arrangement (Plate 17,A&B). Electron-dense material between the cyst and host cell walls was evident (Plate 17,A). Compared with earlier stages of zoospores attached to roots (Plates 15&16), there were many lomasome-like bodies present in the cyst cytoplasm of both *Olpidium* and *Olpidium*/TSA isolates. Lomasome-like bodies were also present between the host cell wall and host plasmamembrane

adjacent to the cyst (Plate 17,A). Two and 3 hours after inoculation cysts were observed with a large vacuole distal to the site of attachment to the host cell wall (Plate 18,A). Lomasome-like bodies were present in the cyst adjacent to the site of attachment. Penetration of the host cell wall by the cyst cytoplasm occurred after 2 hours of encystment on the root surface, as evidenced by penetrating thalli in roots sampled 3 hours after inoculation (Plate 18,B&C). The fungal cytoplasm penetrated the host cell wall and the material deposited between the host plasmamembrane and cell wall (Plate 18,C). Once inside the host cell the fungal cytoplasm was separated from the host cytoplasm by only a single membrane. Empty cysts still contained the cyst plasmamembrane and some lomasome-like bodies inside the cyst wall (Plate 18,B&C). Lomasome-like bodies were also present in the penetration channels. The material deposited between host plasmamembrane and cell wall had a granular matrix and contained some membranous or lomasome-like bodies (Plate 18,B).

No differences were observed in these stages of the two isolates of *Olpidium* examined. Virus or virus-like particles were not observed.

E. FUNGAL THALLI WITHIN HOST CELLS

1. Materials and methods

Tobacco roots were inoculated with zoospores of *Olpidium* and *Olpidium*/TSA, and fixed for examination in light and fluorescence microscopes, SEM and TEM as outlined in section D. Roots were sampled from 4 to 120 hours after inoculation. Some roots containing abundant zoosporangia and resting sporangia were also selected from tobacco

seedlings grown in microincubators, 2 to 4 weeks after inoculation with resting sporangia of *Olpidium* and *Olpidium*/TSA. These roots were fixed for TEM examination as outlined in Chapter III. They were also fixed using the same procedures, freeze-fractured as outlined in section D, and examined with the SEM. Further root samples containing resting sporangia were fixed in 1% aqueous potassium permanganate for 3-4 hours at room temperature, and then washed, dehydrated, embedded and sectioned for TEM examination as outlined in Chapter III.

2. Results

Fungal thalli, 6 to 8 µm in diameter, were visible in roots, sampled 12 hours after inoculation, examined with the light microscope (Plate 6,A). Areas of fluorescence in the same roots were not associated with the fungal thalli (Plate 6,B). The thalli increased in size during the next 12 to 24 hours. At 24 hours after inoculation they possessed a wall and were 10 to 14 µm in diameter (Plate 6,C). At 48 hours, zoosporangia were observed with single exit tubes (Plate 6,D) but no zoospores were released until approximately 72 hours after inoculation. Resting sporangia, each with their characteristic thick undulating wall, were observed for the first time at 96 hours (Plate 6,E), and also elongated zoosporangia with many exit tubes (Plate 6,F).

Freeze-fractured roots examined in the SEM contained fungal thalli within the epidermal cells 4 hours or more after inoculation with zoospores. At 24 hours the thalli were round in outline with a ruffled surface, either due to the presence of host cytoplasm or to the deposition of wall material exterior to the thallus plasmamembrane. Thalli at 48 hours possessed a wall and were surrounded by host cytoplasm which gave them a ruffled surface outline (Plate 11,A&B).

The fungal cytoplasm, as compared with the surrounding host cytoplasm, was very dense at this stage (Plate 11,B). One fractured thallus had an apparent connection with the root surface (Plate 11,C). This hole in the epidermal cell wall may represent the penetration channel for the entrance of the cyst cytoplasm. Zoosporangia with exit tubes were present in epidermal cells at 72 hours (Plate 11,D), and their cytoplasm at this stage contained many small vacuoles (Plate 11,E). At 96 hours mature zoosporangia with exit tubes were observed in the epidermal cells (Plate 12,A), and fractured zoosporangia contained mature zoospores (Plate 11,F). Resting sporangia were observed for the first time in fractured roots at 96 hours after inoculation with zoospores (Plate 12,B&C).

Sections of thalli examined with the TEM possessed only a single limiting membrane for the first 24 hours in the epidermal cells (Plate 19,A). The fungal cytoplasm was dense with ribosomes as compared with the host cytoplasm (Plate 18,C; Plate 19,A). Fungal mitochondria were orthodox in form, whereas those of the host were condensed. At 36 hours, a wall had been deposited exterior to the thallus plasmamembrane (Plate 19,B&C). Some host cytoplasm containing condensed mitochondria was present around the thalli, and the host plasmamembrane was often separated from the host cell wall. The fungal cytoplasm varied in density at this stage and organelles were not distinct. At 48 hours the fungal cytoplasm was multinucleate and contained many vesicles (Plate 19,D). The cytoplasm was then cleaved by these vesicles to differentiate zoospores (Plate 20,A&B). Oblique sections of flagella were present in the cytoplasm which was enclosed by the zoosporangium wall.

This wall appeared to be made up of two layers varying in electron density (Plate 20,A). Mature zoosporangia contained differentiated zoospores and possessed a plugged exit tube (Plate 20,B). These zoospores were limited by a single plasmamembrane, and the ribosomes in different areas of the cytoplasm varied in density (Plate 20,C&D). Axonemal fibrils surrounded by the flagellum membrane were seen in transverse and oblique sections, and the relationship of the flagellum to the kinetosome, rhizoplast and nucleus (Plate 20,D) were the same as for free zoospores (Plate 14,C).

Resting sporangia with characteristic ridges were observed in tobacco roots 96 hours after inoculation with zoospores (Plate 12,B&C). The ridges were more pronounced at 120 hours (Plate 12,D). Resting sporangia were present in the cells of the epidermis and cortex of freeze-fractured roots containing fungus cultures 3 to 4 weeks old. Their distribution was observed in longitudinal and transverse fractures of roots (Plate 12,E&F). No resting sporangia were observed in xylem or phloem cells. The wall of each resting sporangium possessed distinct ridges which outlined five and six-sided facets (Plate 13,A). The ridged walls of freeze-fractured resting sporangia were composed of several layers (Plate 13,B&C), and enclosed the fungal cytoplasm which had a sculptured surface. Two types of sculptured surface were observed; one with raised areas giving a 'raspberry-like' appearance (Plate 13,B), and the other with rod-like depressions (Plate 13,C&D). The paired fractured surface to that shown in Plate 13,C had rod-like raised areas (Plate 13,E&F). In some cells host material was associated with the surface of the resting sporangia (Plate 12,F; Plate 13C).

In the TEM developing resting sporangia had dense cytoplasm containing many organelles which resembled mitochondria (Plate 21, A&B). At an early stage in its development the fungal wall was thick but did not possess the characteristic ridges of mature resting sporangia. The cytoplasm in the host cell had degenerated and the plasmamembrane was dislodged from the host cell wall (Plate 21,B). Resting sporangia with ridged walls contained dense cytoplasm and large bodies of lower electron density after fixation with potassium permanganate (Plate 21,C). The walls were composed of two distinct layers. The inner layer was electron transparent and contained fibrous structures (Plate 21,D). Resting sporangia fixed in aldehydes and osmium also contained dense cytoplasm with many lipid bodies at the periphery (Plate 21,E). The walls were composed of three distinct layers, the inner layer appearing electron transparent except for fibrous-like material (Plate 21,F).

No differences were observed between the isolates of *Olpidium* and *Olpidium*/TSA as regards thalli at different developmental stages within tobacco cells. Virus or virus-like particles were not observed in any *Olpidium*/TSA thalli in host cells.

F. DISCUSSION

Attempts to visualise TSA in different stages of its vector were not successful. The isolates of *Olpidium* and *Olpidium*/TSA were identical as regards life-cycle, morphology and ultrastructure. The ultrastructure of this tobacco *Olpidium* was similar to that of the lettuce and cabbage isolates (Lesemann and Fuchs, 1970a, 1970b; Temmink and Campbell, 1968, 1969a, 1969b; Temmink, 1971). *Olpidium* has a short and comparatively simple life-cycle since one

generation of zoospores is produced in 3 to 4 days. Several zoosporangia may develop in a single host cell and each zoosporangium is capable of releasing many zoospores. Therefore a high probability for transmission of TSA exists, even if only a percentage of the zoospores carry the agent.

In this study the accuracy of timing the development of different *Olpidium* stages was limited by the inability to synchronise the attachment of zoospores to the roots, and also the penetration of the host cells by the fungal cytoplasm. Using concentrated zoospore suspensions (10^8 zoospores/ml) as inocula, sufficient zoospores were attached to the roots after 15 minutes of incubation to permit SEM and TEM examinations of later stages. Observations made with the SEM were extremely valuable for correlating light microscope observations of morphology and TEM observations of ultrastructure of different *Olpidium* stages. Discussion of the morphology and ultrastructure of *Olpidium* will be limited to observations which differ from previous reports, and to unpublished observations.

1. Zoospores free and attached to tobacco roots

Based on light microscope and SEM observations the body of an *Olpidium* zoospore is concluded to be round in outline. Temmink and Campbell (1969a) contended that the body was pyriform from their TEM observations of zoospore sections. However, a later publication (Temmink, 1971) included a light micrograph of a zoospore with a round body. Since the body is limited by only a single plasmamembrane it is suggested that the outline is determined by the environment, and any movement of the body within that medium. In this study sectioned zoospores were frequently not round in outline, due to

external forces involved during processing for embedding and sectioning. Therefore, a conclusion concerning body shape based on sectioned zoospores is not as reliable as that from light microscope and SEM observations.

Zoospores were always observed with a single flagellum. No evidence was obtained for the existence of 'double' or multi-flagellate zoospores (Temmink and Campbell, 1969a; Tomlinson and Garrett, 1964; Garrett and Tomlinson, 1967), which some investigators consider as support for a sexual phase in the life-cycle of *Olpidium* (Kole, 1954; Sahtiyanci, 1962). The ring-like swelling at the base of the flagellum may represent an outline of the kinetosome. Electron micrographs of thin sections of zoospores showed a swelling in the region of the kinetosome, which in previous reports (Temmink and Campbell, 1969a; Temmink, 1971) was briefly referred to as an invagination of the zoospore plasmamembrane.

The fixation and dehydration methods were extremely important for good preservation of zoospores free or attached to tobacco roots for SEM examination. Prior to the development of the cyst wall the zoospore body collapsed easily during preparation unless osmium was used for fixation. Freeze-drying resulted in damage and collapse compared with the more gentle dehydration by the critical point method.

Zoospores were firmly attached to the roots prior to retraction of the flagellum, and were not washed off during processing as previously reported (Temmink, 1971). Observations of zoospores with the flagellum partially retracted and ridge formation suggest that retraction occurs when the flagellum wraps around the body. This also agrees with earlier light and electron microscope observations

(Koch, 1968; Lesemann and Fuchs, 1970a). In the present study merging of the body and flagellum membranes was apparent where the flagellum was wrapped around the body. Merging may occur along the length of the flagellum simultaneously, or it may be gradual starting from the base of the flagellum, as evidenced by micrographs showing part of the flagellum still free. This may be related to the time required for the flagellum to be wrapped around the zoospore body.

Electron micrographs of free zoospores showed the body and flagellum membranes to be continuous. Thus the flagellum membrane was probably incorporated into the body membrane during retraction. This may have occurred in a manner similar to that suggested for cell membrane fusion and the fertilisation mechanism in plants and animals (Friedmann, 1962). Comparable fusion of membranes has been demonstrated during the resorption of cilia by certain protozoa (Roth and Shigenaka, 1964), and the fusion of isolated plant protoplasts (Power *et al.*, 1970). The stimulus for merging of the flagellum and body membranes must result from at least 2 factors, i.e. adhesion of the zoospore body to the root surface, and wrapping of the flagellum around the body which brings the membranes into close proximity, a prerequisite for membrane fusion (Lucy, 1970; Poste and Allison, 1973). Adhesion seems to be a requirement since free zoospores do not show this merging of the membranes. Further factors must also be involved since merging does not occur between the flagellum and body membranes of different zoospores attached to roots.

Prior to encystment the zoospore cytoplasm is limited by only a single plasmamembrane which may have 'elastic' properties.

Zoospores attached to roots but with the flagellum still present had an irregular body outline. Likewise those with the flagellum partially retracted also had a body outline which was irregular and flattened. This irregular folding of the plasmamembrane is similar to observations reported for zoospores of *Blastocladiella emersonii* undergoing encystment (Truesdell and Cantino, 1971). A discoid or flattened shape of zoospores immediately following retraction of the flagellum has also been reported for the chytrid fungus, *Rozella allomycis* (Held, 1973) and for *Plasmodiophora brassicae* (Aist and Williams, 1971). The flagellum of a zoospore of *R. allomycis* is retracted when it wraps around the stationary body of the zoospore (Held, 1973). In contrast the flagella of a *P. brassicae* zoospore are retracted when they become coiled (Aist and Williams, 1971). With both these fungal zoospores the appearance of a discoid cyst with an irregular outline at the time of flagellum retraction has been demonstrated by light microscope and TEM observations. In the present study the same discoid shape of the cyst of *Olpidium* has been observed immediately following flagellum retraction. The area of contact between the cyst and the host cell wall becomes reduced as the cyst rounds off with the deposition of the cyst wall. The discoid shape of the cyst and irregular folding of the plasmamembrane may result from a spring-like coil of the axoneme (Held, 1973), or from other forces involved in retraction since free and encysted zoospores after the same fixation procedures did not display this characteristic. Following retraction of the flagellum, cyst wall material was rapidly deposited exterior to the plasmamembrane, and this cyst wall may account for increased resistance of the otherwise originally delicate

zoospore body prior to penetration of the host.

The number of zoospores attached to roots and still possessing an entire or a partially retracted flagellum was low relative to the number of attached zoospores at any given time. This suggests that flagellum retraction occurs rapidly after attachment of the body to the root. Because of the extremely low distribution of zoospores showing stages of flagellum retraction at any given time, SEM had practical advantages in this study over sectioning for TEM for observation of these stages. If a system could be developed in which the zoospores were induced to attach to the root cells within a very short time interval, then a more detailed investigation of these prepenetration stages using TEM and SEM would be possible.

During the period of encystment on the root surface the increased number of membrane-bound vesicles or lomasome-like bodies in the cyst cytoplasm may be involved in the deposition of the cyst wall (Lesemann and Fuchs, 1970a; Manton, 1964; Temmink and Campbell, 1969b; Truesdell and Cantino, 1971), or in the development of a large vacuole distal to the site of attachment to the host cell wall (Held, 1973 ; Temmink and Campbell, 1969b). Lomasome-like bodies between the cyst wall and plasmamembrane adjacent to the host cell wall may function as a store of membranous material (Heath and Greenwood, 1970) for the penetrating thallus. However, the presence of similar bodies in the cyst after passage of the cytoplasm into the host cell may indicate a surplus of membranous material during penetration. Lomasome-like bodies between the host plasmamembrane and cell wall were probably involved in the deposition of callose substances and the thickening of the wall adjacent to the cyst. This modification

of the host wall appeared before penetration by the fungal thallus, as evidenced by TEM and SEM observations of encysted zoospores. Similar timing of the host wall modification has been reported for several host-parasite combinations (Edwards and Allen, 1970; Hanchey and Wheeler, 1971; Temmink and Campbell, 1969b).

The presence of callose substances in the cell wall modification induced by *Olpidium* cysts was demonstrated by bright fluorescence under UV illumination after staining with aniline blue (Eschrich and Currier, 1964). The exact chemical nature of this cell wall modification is unknown since the specificity of aniline blue fluorochrome for different glucans is uncertain (Faulkner *et al.*, 1973). Smith (1900) referred to this structure as the "papilla". Since then it has been called "lomasomes" (Ehrlich *et al.*, 1968), "papillum" (Temmink and Campbell, 1969b), and "callosity" (Lesemann and Fuchs, 1970b). This structure is thought to be produced by the host protoplast (Aist and Williams, 1971; Bushnell, 1971; Chou, 1970; Edwards and Allen, 1970; Ehrlich *et al.*, 1968; Kusano, 1936; Lesemann and Fuchs, 1970b), and comparable cell wall modifications have been reported during the penetration of plant cells by other fungi (Aist and Williams, 1971; Berlin and Bowen, 1964; Ehrlich *et al.*, 1968; Hardwick *et al.*, 1971; Heath and Heath, 1971; Mercer *et al.*, 1975; Peyton and Bowen, 1963; Politis and Wheeler, 1973; Sargent *et al.*, 1973). Since the wall modification occurs before penetration by the *Olpidium* thallus it is suggested that this is a host response which serves as a defense mechanism against penetration (Heath and Heath, 1971). In comparison, papillae produced in cells penetrated by zoospores of *P. brassicae* (Aist and Williams, 1971) were concluded to serve as

a localised wound-healing response of the host (Currier, 1957; Nims *et al.*, 1967). Callose substances can probably be regarded as a non-specific response to penetration. Callose itself is stated to be electron-lucent and non-fibrous in texture (Frey-Wyssling and Muhlethaler, 1965; Heslop-Harrison, 1966). The wall thickening during *Olpidium* penetration is slightly granular and contains membranous bodies, suggesting that it is composed of other materials as well as callose.

The actual mode of host cell wall penetration by *Olpidium* zoospores is not known. In the absence of any specialised structure for piercing the wall as found in other Phycomycete fungi (Aist and Williams, 1971; Keskin and Fuchs, 1969), it is suggested that penetration may be mainly enzymatic since no conspicuous signs of stress were observed in the wall. The lomasome-like bodies in the cyst at the site of attachment to the host cell wall may contain wall degrading enzymes (Lesemann and Fuchs, 1970a). However, in the case of cysts of *P. brassicae*, which contain a specialised structure for physical penetration of the host wall, it has been proposed that these lomasome-like bodies contain enzymes which strengthen the adhesive material between the cyst and host cell wall (Aist and Williams, 1971).

2. Thalli within host cells

During the process of penetration, the cyst cytoplasm passes through both the host cell wall and the plasmamembrane. The fungal thallus is then in close contact with the host cytoplasm, separated by only a single membrane which is thought to be the thallus plasma-membrane (Temmink and Campbell, 1969b). This boundary is simple by comparison with the complex haustorial-host boundaries of the rusts

and mildews with their sheath membrane, sheath, haustorial wall, and plasmamembranes (Bracker, 1967), and even with the seven-layered plasmodial envelope of *P. brassicae* (Williams and McNabola, 1970). This stage in the life-cycle of *Olpidium* may be the time when acquisition and transmission of TSA occurs. Exchange of materials may occur between the tobacco host and *Olpidium* at this time, whereas after the deposition of the thallus wall, approximately 24 hours after penetration, the host and fungal cytoplasm are physically separated. Following the formation of the thallus wall the host cytoplasm appears degraded, whereas the fungal cytoplasm undergoes differentiation as previously reported (Temmink and Campbell, 1968, 1969b).

The factors controlling resting sporangia development are still unknown. Resting sporangia are present in the same cells as zoosporangia, however, the former frequently occur in different layers of the cortex, whereas the latter are restricted mainly to the epidermis and first layer of the cortex. It is possible that the immediate environment of a fungal thallus determines its differentiation, since the presence or absence of certain ions has been shown to influence the formation of resting sporangia by *Blastocladiella emersonii* (Griffin, 1965). The distribution of *Olpidium* thalli in different layers of the cortex raises the intriguing question of how they reach the cells inside the root. Zoosporangia in epidermal cells have been observed to release zoospores within the cell. These zoospores may encyst on the wall between an epidermal and cortical cell, as evidenced by cyst-like structures adjacent to cell wall thickenings observed with the TEM. The absence of the host cytoplasm and plasmamembrane at this stage would suggest that the 'internal'

zoospores were able to encyst on the 'internal' cell wall. However, it is not known whether this represents penetration by secondary 'internal' zoospores (Aist and Williams, 1971) into the first layer of the cortex, or whether the fungal cytoplasm present in the epidermal cell encysts directly and penetrates into the cortex cells, without the formation of a zoosporangium and a new generation of zoospores.

The differentiation of thalli into zoosporangia or resting sporangia is apparent approximately 48 hours after penetration. The cytoplasm of thalli developing into resting sporangia does not become multinucleate. Organelles in the cytoplasm appear to break down, and there is an increase in lipid bodies. Large quantities of lipid material have also been reported inside resting sporangia of *P. brassicae* (Williams and McNabola, 1967), a *Chytridium* species (Schnepf *et al.*, 1971) and chlamydospores of *Fusarium oxysporum* (Griffiths, 1973). In *Olpidium* resting sporangia the lipid bodies appear at the periphery of the cytoplasm, and may produce the undulations on the surface of the cytoplasm as observed in freeze-fractured samples with the SEM. Resting sporangia of a *Chytridium* sp. also possess lipid bodies at the periphery of the cytoplasm (Schnepf *et al.*, 1971). The wall of an *Olpidium* resting sporangium is laid down exterior to the thallus plasmamembrane, and appears amorphous initially. However, when the wall ridges have developed, several layers of the wall are evident. The two outer layers are electron-dense and fibrillar. The 5 and 6-sided facets delineated by the wall ridges probably permit contraction and expansion of the resting sporangium according to environmental conditions. Air-dried resting sporangia

possess facets which are more deeply sunken than those of resting sporangia inside host cells.

The persistence of TSA in *Olpidium* infested soil is explained by the internal association of the agent with resting sporangia. These resting sporangia are able to withstand unfavorable environmental conditions due to their thick multi-layered wall, and remain viable for long periods of time. The increase in lipid bodies in the cytoplasm of mature resting sporangia, and concomitant decrease in organelles, suggest a dormant condition of these stages of *Olpidium*.

The absence of any conventional virus particles in all stages of *Olpidium*/TSA suggests that there may be a close association between the nucleic acids of TSA and its vector, or some other cellular component of *Olpidium*. This would indicate an extremely stable relationship between agent and vector. No evidence was obtained to support the report of particles in *Olpidium*/TSA zoospores (Soejima and Hidaka, 1969). Since it is possible to free the fungus of TSA by culturing on cowpea, *Vigna sinensis* (Hiruki, 1965), it is thought that TSA is not capable of multiplication in its vector.

CHAPTER VII

HISTOLOGICAL AND CYTOLOGICAL ABERRATIONS FOLLOWING INFECTION OF TOBACCO WITH *OLPIDIUM* AND TOBACCO STUNT AGENT

A. INTRODUCTION

Infection of tobacco with TSA, following inoculation of the roots with *Olpidium*/TSA, produces severe disease symptoms which are typical of virus and 'yellows' diseases. The external disease symptoms (Hidaka *et al.*, 1956) do not provide a reliable indication of the identity of the causal agent, except to further suggest that it is distinct from tobacco necrosis virus (TNV). The sites of histological and cytological aberrations in diseased plants provide an indication of possible causal agents (Schneider, 1973), although similar histological aberrations can be produced by diverse causal agents. To date the changes which occur in tobacco tissues and cells following stunt infection have not been reported. In this study, infected tobacco tissues were examined for the following reasons:

1. to determine the effects of *Olpidium* and TSA separately as regards symptom expression.
2. to attempt visualisation of TSA in different tobacco tissues.
3. to determine the significance of vascular necrosis in stunted tobacco.

The external symptoms of stunt disease previously reported were based on observations of tobacco plants grown in infested soil

(Hidaka *et al.*, 1956), and also after sap inoculation with TSA (Hiruki, 1964, 1967, 1975). Hidaka *et al.* (1956) briefly reported necrosis in the vascular tissues of tobacco stems and leaves showing stunt symptoms. However, these observations were made from plants grown in infested soil. No examinations have been made of vascular tissues in stunted tobacco in the absence of *Olpidium*. It is therefore important to evaluate the roles played by *Olpidium* and TSA separately in the development of external symptoms, and to relate this to histological aberrations in stunted tobacco.

In view of the uncertainty as to the nature of TSA it is necessary to examine infected tissues for the presence of such micro-organisms as mycoplasmas and rickettsias. This is especially relevant because of the vascular necrosis previously reported (Hidaka *et al.*, 1956). Virus-like agents causing certain aberrations in the phloem tissues of plants are now known to be a mixture of conventional viruses and mycoplasmas. Similarly, agents which produce virus-like symptoms in the xylem and were thought to be viruses, now appear to be rickettsias (Schneider, 1973).

Fluorescence microscopy has been used for the detection of phloem cells containing mycoplasma-like organisms in diseased plants (Dijkstra and Hiruki, 1974; Goszdziewski and Petzold, 1975; Hiruki and Dijkstra, 1973; Hiruki *et al.*, 1974b). The presence of mycoplasma in phloem sieve cells is accompanied by high quantities of callose around the cell walls, and necrosis of cells. This abnormal deposition of callose can be detected after staining with aniline blue fluorochrome and examining with the fluorescence microscope.

Scanning electron microscopy has been used for the examination

of the internal structure of biological material (Bole and Parsons, 1973; Germinario and McAlear, 1971), and provides the advantage of higher resolution when compared with the light microscope. This technique was included in the present study for observations of histological aberrations in healthy, *Olpidium*-infected and TSA-infected tobacco plants.

B. EXTERNAL SYMPTOMS OF *OLPIDIUM* AND TSA INFECTIONS

1. Materials and methods

a) Inoculation of tobacco with *Olpidium*/TSA.

Tobacco seedlings were inoculated with zoospores of *Olpidium* and *Olpidium*/TSA as outlined in Chapter III, and grown at 18° ± 2°C for 3 months. The development of any disease symptoms was recorded. Healthy, non-inoculated plants were also grown as controls.

One week old tobacco seedlings growing in sand in pot incubators (Plate 1,A) were inoculated with zoospores of *Olpidium* and *Olpidium*/TSA, and grown at 18° ± 2°C for 2 months. Roots were periodically sampled and examined after washing out the sand with water.

b) Sap inoculation of tobacco with TSA.

Inoculations were carried out as outlined in Chapter III. Inoculated plants and non-inoculated controls were grown as in a).

c) Graft transmission of TSA to healthy tobacco.

Shoots of tobacco showing symptoms of stunt, 4 weeks after inoculation of the roots with *Olpidium*/TSA zoospores, were wedge-grafted onto healthy 6 weeks old tobacco plants. The graft unions

were bound with parafilm and the plants covered with polyethylene bags for 1 week. After removal of the bags, the plants were grown for 3 months at 18° ± 2°C, and the development of any symptoms of stunt infection on the new growth from the stocks was recorded. Shoots of healthy and *Olpidium* inoculated tobacco were also grafted as controls.

2. Results

a) Tobacco inoculated with *Olpidium*/TSA.

The first symptoms of stunt appeared as water-soaking of small scattered areas towards the tip of young leaves, approximately 3 weeks after inoculation. One day later these areas had become necrotic, but remained about the same size. Vein-clearing of young expanding leaves was also evident at this time. These young leaves were more erect than comparable ones in the healthy and *Olpidium* inoculated controls. During the next 3 days fine chlorotic and necrotic spots developed extensively over the leaves. Some leaves had ring-like and wavy patterns of chlorosis and necrosis. Approximately 4 weeks after inoculation the plants appeared stunted and possessed short internodes with the leaves in a rosette arrangement. Developing leaves of stunted plants were smaller than those of the controls, and were distorted in outline (Plate 3,B). Plants with severe symptoms of stunt had small irregularly shaped leaves in a rosette arrangement, and the lower leaves at the base of the plant were stiff and brittle. Considerable necrosis developed along leaf veins, petioles and stems. A ring-type necrosis occurred on the stem at soil level (Plate 2,D). This development of vascular necrosis was accompanied by wilting of

the plants during the daytime.

No disease symptoms developed on the leaves of control plants. However, there was a difference between the growth rates of healthy and *Olpidium* inoculated controls during the first month after inoculation. The growth of *Olpidium* inoculated plants was retarded compared with the healthy controls. But at 2 months after inoculation there was no apparent difference between these control treatments (Plate 3,A). However, the difference between the growth of *Olpidium*/TSA inoculated plants and the 2 controls was very distinct. Root development of plants from the different treatments was compared after washing soil out of the roots with water. The root systems of healthy and *Olpidium* inoculated control plants were well developed and light in colour in contrast to the roots of *Olpidium*/TSA inoculated plants (Plate 2,D). The latter roots were poorly developed and brown in colour. Also, severe stem necrosis was observed on these plants at soil level. The stems of control plants were normal in appearance.

The tobacco seedlings grown in sand culture and inoculated with zoospores of *Olpidium* and *Olpidium*/TSA developed small chlorotic spots on the seed leaves, approximately 10 days after inoculation. No symptoms were observed on healthy controls. Seedlings inoculated with *Olpidium*/TSA developed typical symptoms of stunt infection after incubating for a further 10 days, whereas no stunt symptoms developed on the *Olpidium* inoculated controls.

The washed roots of *Olpidium* and *Olpidium*/TSA inoculated seedlings were brown in colour and poorly developed, when compared with the roots of healthy controls, during the first month after inoculation. Subsequently, the root development and root colour of

Olpidium inoculated seedlings were comparable to those of the healthy controls. However, the roots of *Olpidium*/TSA inoculated seedlings still appeared brown and stunted in their development.

b) Tobacco, sap inoculated with TSA.

Following sap inoculation, the first symptoms developed on the inoculated leaves after incubating for 6 to 8 days. Typical ring necrosis (Plate 2,A) and fine necrotic spots appeared first as chlorotic areas, and then became necrotic during the next 2 days. Also some of these areas enlarged and fused later. Systemic symptoms were first observed in young expanding leaves, 12 days after inoculation. Vein-clearing in these leaves was followed 2 days later by systemic necrosis (Plate 2,B&C). Stunting of the plants (Plate 1,D) occurred 6 to 7 days later. However, not all inoculated plants developed symptoms of stunting. In one set of inoculations, 17 out of 24 plants inoculated developed stunting symptoms. The remaining plants developed only local and systemic necrosis. Necrosis of the stem at soil level was only observed in plants exhibiting stunting symptoms (Plate 1,E). After incubation for more than one month the stunting was severe, with short internodes and small irregularly shaped leaves produced. Severe necrosis developed along leaf veins, petioles and stems, and the lower leaves were stiff and brittle.

c) Graft transmission of TSA to tobacco.

Two weeks after grafting infected scions onto healthy tobacco stocks, symptoms of systemic necrosis were observed on new growth from the stocks. These shoots were stunted and had short internodes (Plate 1,C). Symptoms were identical with those produced after sap or

Olpidium transmission of TSA. No symptoms developed on the tobacco plants grafted with healthy and *Olpidium* inoculated controls.

C. HISTOLOGICAL AND CYTOLOGICAL ABERRATIONS

1. Materials and methods

Tissue samples were taken from the same plants as examined in section B, i.e. tobacco plants showing symptoms of stunt infection following sap inoculation with TSA, graft and *Olpidium* transmission of TSA. Tissue samples were also taken from healthy and *Olpidium* inoculated control plants. All samples were processed and examined as outlined in the following sections a), b) & c).

a) Light and fluorescence microscopy

Fresh, hand-cut sections of stems and petioles were stained for lignins, tannins, callose and gums (Esau, 1948; Eschrich and Currier, 1964; Jensen, 1962), using the following procedures:

(i) Sections were mounted in a saturated aqueous solution of phloroglucinol in 20% hydrochloric acid. Lignin stained a red-violet colour.

(ii) Sections were bleached in saturated acidified calcium hypochlorite for 5 minutes, and then mounted in 1% sodium sulphite. Lignin stained a bright red colour, which faded to brown after 40 minutes.

(iii) Sections were mounted in a 1% solution of ferric chloride in 0.1 N hydrochloric acid. A blue stain indicated the presence of tannins.

(iv) Sections were placed in a glass dish and equal volumes of the following reagents added: 10% sodium nitrate, 20% urea and 10% acetic acid. After leaving for 3 to 4 minutes, 2 volumes of 2N sodium hydroxide were added. Tannins stained a cherry red colour.

(v) Sections were fixed in boiling water for 5 minutes, mounted in aniline blue stain, and then examined with the fluorescence microscope (Chapter III). Callose substances fluoresced a bright yellow-green colour (Eschrich and Currier, 1964).

(vi) Sections were stained with 0.5% aqueous safranine O for 1 minute, and then washed in 4 changes of distilled water. Cell walls were stained purplish red, and gum materials stained scarlet.

The roots of tobacco seedlings grown in sand culture and inoculated with *Olpidium* and *Olpidium*/TSA zoospores, as in section B, were also examined periodically with the light microscope. Washed roots were mounted in water and examined without fixation.

b) Scanning electron microscopy

The following schedules were used for the preparation of tobacco stems and petioles for SEM examination:

(i) Stem and petiole sections, 1-2 mm thick, were cut with razor blades and frozen in liquid Freon 13. After transferring into liquid nitrogen they were freeze-dried.

(ii) Sections were washed in 3 changes of distilled water and freeze-dried.

(iii) Sections, 5 mm thick, were fixed overnight at 4°C in 0.1 M phosphate buffer pH 7.0 containing 2% glutaraldehyde plus 2% formaldehyde. The tissues were washed twice for 1 hour in distilled water,

and postfixed for 6 hours in 2% aqueous osmium tetroxide at room temperature. After washing again for 1 hour, sections 1-2 mm thick were cut from the centre of each piece of tissue, and freeze-dried.

Dried sections were mounted for SEM examination as outlined in Chapter III.

c) Transmission electron microscopy

Tissue pieces, 2 to 3 mm square, were cut from stems, leaves and petioles of tobacco plants showing stunt symptoms, following sap inoculation with TSA, and graft and *Olpidium* transmission of TSA. Tissue samples were also taken from healthy and *Olpidium* inoculated control plants, and from leaves of *Chenopodium amaranticolor* with 3 days old local lesions following sap inoculation with TSA (Chapter III). All tissue samples were fixed, dehydrated, embedded and sectioned for TEM examination as outlined in Chapter III.

2. Results

a) Light and fluorescence microscopy

A regular arrangement of xylem and phloem tissues was observed in stem sections of healthy and *Olpidium*-inoculated controls (Plate 22, A&B, C&D). In the fluorescence microscope the lignified walls of the xylem autofluoresced very strongly. This fluorescence which was very regular and light blue in colour, also appeared in the thick walls of sclerenchyma cells. Specific yellow-green fluorescence was observed as regularly distributed spots in the phloem tissues after staining callose with aniline blue fluorochrome. The phloem tissues were small in area and fluorescence was associated with the sieve plates, as seen in longitudinal sections (Plate 24,A&B).

In contrast, the vascular tissues in stems and petioles of plants infected with TSA were highly modified (Plate 23,A&B). Cells of the xylem were irregular in arrangement and had walls of varying thickness. Fluorescence was irregular and depended on the degree of lignification of the walls. Thin walled cells fluoresced very faintly as compared with healthy xylem cells. In longitudinal sections, the weak autofluorescence in the xylem was associated with vessels which were very short in length and irregular in arrangement (Plate 24,C&D). Hyperplasia was evident in the phloem tissues in stems and petioles of stunted tobacco, but no virus or microorganisms were observed in the cells. These cells fluoresced with a higher intensity than corresponding phloem cells in healthy stems, but the distribution of the fluorescence was normal. This was also particularly evident from longitudinal sections of stems. The sieve cells were very short, and fluorescence was limited to the areas of the sieve plates (Plate 24,C&D). Sclerenchyma cells were present in tobacco stems showing stunt symptoms, but were absent in stems with severe symptoms of stunt.

Tobacco stems and petioles after sap inoculation with TSA (Plate 23,C&D), and also after graft transmission with TSA, had irregularly thickened cell walls in the xylem. Again, hyperplasia was evident in the phloem, but the fluorescence in these cells was normal in distribution.

No tannins were detected in the tissues of stunted tobacco or in the controls. Staining for lignin showed the irregular distribution of thick walled xylem cells in stunted tobacco. Also the walls stained with different intensities as compared with the more uniform

staining of healthy xylem walls. Sections from stunted tobacco, stained with phloroglucinol and with safranine, showed material present in the xylem vessels. This material, which stained red, did not occur in the stem sections of healthy and *Olpidium*-inoculated controls.

Tobacco roots heavily infected with *Olpidium* and *Olpidium*/TSA were stunted, and contained areas of light brown discolouration throughout the cortex. The root tips were stubby in appearance, and cells in the zone of cell elongation were crowded together. Many root hairs were swollen at the tip but did not contain any visible fungal thalli. *Olpidium* was distributed in cells of the epidermis and cortex.

b) Scanning electron microscopy

The surfaces of stem and petiole sections, freeze-dried without any prior treatment, were covered with material from the cut cells. This was particularly evident for stem sections of stunted tobacco. Washing the sections in water before freeze-drying was sufficient to remove this material. This latter procedure permitted examination of the distribution of different tissues and the cells with these tissues, especially the xylem and phloem.

In stem sections of healthy and *Olpidium*-inoculated plants, the distribution of the vascular tissues was normal. The ring of thick walled xylem vessels was surrounded by small groups of cells constituting the internal and external phloem (Plate 25,A&B, C&D). The vascular tissues in sections of stunted tobacco stems were extensively developed, but the cells within such tissue were irregular in distribution (Plate 25,E&F). In contrast with the xylem cells

of healthy and *Olpidium*-inoculated controls (Plate 26,A&C), xylem cells in stunted tobacco were thin-walled (Plate 26,B&D). Large xylem vessels were infrequent in stunted tobacco, especially after the development of symptoms of severe stunting. The difference between the lignified cell walls in *Olpidium*-inoculated tobacco xylem and stunted tobacco xylem was particularly evident at high magnification (Plate 26,C&D). Hyperplasia of phloem tissues was evident in stunted tobacco (Plates 25,F & 26,F) especially when contrasted with the phloem in healthy tobacco (Plate 26,E). However, no necrosis was observed in the phloem cells of stunted tobacco.

Gum-like material was detected in the sections of stunted tobacco petioles (Plate 27,A) which showed external symptoms of necrosis. This material appeared to merge with the thin walls of the xylem (Plate 27,B). However, upon closer examination, it was revealed that this material was actually on the cut surface of the sections (Plate 27,C), and had probably flowed out of the xylem cells during preparation for SEM examination. This material was also present in the xylem of stunted tobacco stems, but in smaller quantities (Plate 27,D). Stems and petioles of control plants did not contain this material. The sections, which were cut from stunted tobacco and fixed prior to final cutting and freeze-drying, did not show this material on the cut surfaces (Plate 27,E). Instead, this gum-like material was present in necrotic cells in the xylem (Plate 27,F).

c) Transmission electron microscopy

The phloem tissues of stunted tobacco stems showed apparent

hyperplasia (Plate 28,A), but their distribution in transverse sections was regular. Companion cells were present with sieve cells. No necrotic cells were observed. Likewise, no virus-like particles, mycoplasma-like bodies, rickettsias, or abnormal depositions of callose were detected in the phloem cells. Sieve cells contained abundant P-protein and plastids (Plate 28,B). The latter were more numerous in sieve cells of stunted tobacco than in the sieve cells of control plants.

Xylem cells of stunted tobacco (Plate 29,B) were highly degraded when compared with those of the control plants (Plate 29,A). Some xylem vessels possessed lignified walls, but adjacent cells had extremely thin walls which were irregular in outline. The cytoplasm in these cells was degraded. Distinct cytoplasmic organelles visible in the cytoplasm of healthy cells were absent in these thin walled cells.

In tobacco leaves showing stunt symptoms, the chloroplasts were swollen with very large starch granules (Plate 29,C). In some chloroplasts the membrane system appeared to be degraded. In contrast, the starch grains in chloroplasts of healthy tobacco leaves were small and did not disrupt the membrane system. In leaves of *Chenopodium amaranticolor* showing necrotic lesions following sap inoculation with TSA, the chloroplasts were similarly deformed, and the membrane system disrupted, by the presence of large starch grains (Plate 29,D). Necrotic cells were observed in leaves of both tobacco and *C. amaranticolor* infected with TSA. No virus or virus-like particles were detected in these cells or in adjacent cells. There was, however, an increased incidence of lomasome-like

bodies in the leaf cells of tobacco showing stunt symptoms, when compared with similar tissues of the control plants. These bodies were observed between the wall and plasmamembrane of cells, and sometimes the plasmamembrane was deeply invaginated into the cell vacuole. However, the adjacent cytoplasm often appeared normal.

D. DISCUSSION

The external symptoms of tobacco stunt disease are typical of a virus infection as regards the vein-clearing of young leaves, the systemic necrosis of leaves and stems, and the stunting of the whole plant. However, vein-clearing is also observed in plants infected with mycoplasmas, and stunting or reduced vigour has been reported for many plant diseases suggested to be caused by bacteria and rickettsia (Teakle *et al.*, 1973; Hopkins and Mortensen, 1971). The internal symptoms of stunt infection cannot be compared directly with any published reports. The major aberrations occurred in the xylem tissues. Esau (1948) investigated the anatomic effects of Pierce's disease in grapevines which, at one time believed to be caused by a virus, was recently reported to be caused by a bacterium (Auger et al., 1974). Gum-like material was detected in the xylem cells, and the xylem walls remained lignified. In the present study, gum-like material was detected in xylem cells of tobacco showing stunt symptoms, but the walls of these cells were thin and apparently not lignified. It is not known whether the thickening of the xylem cell walls is lost during infection with TSA, or whether lignification of developing xylem cells is prevented in some way. The latter explanation is supported by the

fact that stunting of infected plants is influenced by temperature (Hidaka and Hiruki, 1958). In the present study (Chapter IV), tobacco plants inoculated with *Olpidium*/TSA and incubated at 25°C grew without developing any stunt symptoms. These plants developed stems with normal internodes. However, after incubating at 17°C for 2 to 3 weeks, stunt symptoms appeared at the top of these plants. Examination of the vascular tissues in different parts of the stems showed that the xylem cells had thin walls only in the stunted region of the stems. In lower parts of the stems the xylem cells had lignified walls.

Histological aberrations were mainly restricted to the xylem of infected plants. These changes may explain the wilting of severely stunted plants, since water movement in the plants would be affected. From the combination of light microscopy, SEM and TEM, it was shown that, although hyperplasia of the phloem occurred in stunted tobacco, the cells in these tissues were normal in appearance. The high number and short length of individual phloem cells may have been due to the large number of leaf traces present in a stunted tobacco stem. In this investigation fluorescence associated with callose in the phloem of stunted tobacco plants was not typical for plants infected with mycoplasmas (Dijkstra and Hiruki, 1974; Goszdziewski and Petzold, 1975; Hiruki and Dijkstra, 1973). Also the absence of such microorganisms from the phloem cells suggests that mycoplasmas are not involved in tobacco stunt disease.

The spatial relationships of cells and tissues in stems and petioles of tobacco plants were clearly demonstrated with the SEM. Aberrations in tissues of stunted tobacco were detected using both

light microscopy and SEM. The absence of any microorganisms in the xylem of stunted plants suggests that the gum-like material in these cells may have arisen from the breakdown of the walls or some other host material (Beckman, 1964). The accumulation of starch in stunted plants may also explain the presence of gum-like material in the xylem, since starch has been proposed as a precursor for gum materials (Esau, 1948).

This study has demonstrated that *Olpidium* is not involved in the external and internal symptomatology of tobacco stunt disease, although *Olpidium* infection of very young tobacco seedlings affected the growth of these plants during the first month. Since the cytoplasm of root cells containing *Olpidium* thalli underwent degradation (Chapter VI), it is possible that the root physiology was altered by high infection. However, this effect of *Olpidium* on the growth of the plants was only temporary.

Histological and cytological aberrations were observed in stunted tobacco following sap or graft transmission of TSA. These observations strongly suggest that stunt is induced by TSA alone, and that *Olpidium* is only involved in the transmission of the disease agent to a suitable host plant.

The changes observed in the chloroplasts of stunt infected tobacco and *C. amaranticolor* leaves were characteristic of virus infected cells (Carroll and Kosuge, 1969; Esau, 1967, 1968). This abnormal starch accumulation explains the stiff and brittle nature of stunt infected tobacco leaves.

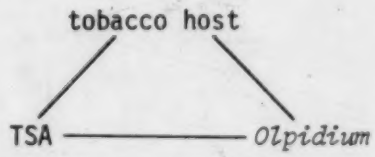
All attempts to visualise virus and virus-like particles, mycoplasmas, rickettsias and bacteria in different cells and tissues

of TSA infected tobacco proved unsuccessful. Since high infectivity of TSA has been recovered from sap obtained from leaf tissues (Chapter V), it should be possible to locate virus particles in these tissues if TSA possesses conventional particles. The apical region of stunted tobacco plants is another area which requires thorough investigation in attempts to visualise TSA. The aberrations in xylem cells may be due to an effect of TSA on differentiating cells in the apical region of tobacco stems.

CHAPTER VIII

GENERAL DISCUSSION

If the 3 biological entities involved in tobacco stunt disease are considered as the points of a triangle, the inter-relationships between the disease agent, fungus vector and host plant can be more easily assessed.



Even though it was suggested that not all zoospores carry TSA (Chapter IV), *Olpidium* can still be considered as an efficient vector. This fungus is parasitic on the roots of tobacco, and does not affect the internal and external symptoms of TSA infection (Chapter VII). This observation supports the idea proposed by Grogan and Campbell (1966) that a fungus with a host-parasite relationship would more likely be a virus vector than a highly pathogenic fungus. Certain characteristics of *Olpidium* enhance its role as vector of TSA. The motile zoospores are the actual vectors which migrate between the roots of diseased and healthy tobacco. The tobacco root cells may each contain several fungal thalli, which mature in only 3 or 4 days and release many zoospores. The production of high numbers of zoospores enhances the probability of successful TSA transmission.

Tobacco root cells reacted to penetration by encysted zoospores with the deposition of callose materials (Chapter VI), and this response was considered to be non-specific. Very recently this deposition of materials was reported to prevent the infection of Kohlrabi root cells by some encysted *Olpidium* zoospores (Aist and Israel, 1975). Following penetration of the host cell wall and plasmamembrane, the fungal cytoplasm was only separated from the host cytoplasm by a single membrane (Chapter VI). This relationship between host and vector probably represents the stage when acquisition and transmission of TSA occurs. The morphology and ultrastructure of resting sporangia gave an indication of their ability to survive adverse conditions. The multilayered wall was very thick and the undulating surface with 5 and 6-sided facets would permit expansion and contraction of the structure according to moisture conditions. The abundant lipid bodies within the cytoplasm suggest the dormant condition of resting sporangia.

It is interesting to compare the stability of TSA in its vector and in the tobacco host. *In vivo* acquisition by *Olpidium* suggests that TSA is carried internally by the fungus (Chapter IV). The stability of TSA inside its vector is demonstrated by the retention of infectious TSA in air-dried resting sporangia for many years. This stable and persistent relationship was further suggested when TSA survived chemical and physical treatments to resting sporangia as long as the fungus remained viable (Hiruki, 1972). Virus-like particles were not detected in *Olpidium* carrying TSA (Chapter VI), therefore the stable association with its vector may possibly be due to an association between the TSA-RNA with components of the cytoplasm or nucleus of *Olpidium*.

In contrast, attempts to isolate TSA from infected tobacco showed that it is extremely unstable in its plant host (Chapter V). The unstable nature of TSA was also shown by longevity, dilution end point and thermal inactivition point in sap. Results indicating that infectious TSA contains RNA provoke questions concerning the relationship of this agent with host cells. Since TSA-RNA is unstable it may be totally or partially single-stranded. In contrast, infectious RNA's, 'viroids', isolated from plants infected with potato spindle tuber and chrysanthemum stunt are stable in plant sap, especially if prepared in high molarity buffer or in extracts treated with phenol (Raymer and Diener, 1969; Diener and Lawson, 1973; Hollings and Stone, 1973). The data obtained in this investigation suggest that TSA is dissimilar to 'viroids'.

In plant cells TSA may exist at certain stages with a loose protein coat, as evidenced by the actions of protease and bentonite on infectivity in sap, precipitation by PEG, fractionation during SDG centifugation, and the presence of many uniform subunit-like particles in non-infectious nucleoprotein preparations (Chapter V). The association of a protein component with TSA is also supported by the recent report of a specific antiserum developed for TSA (Hiruki, 1975). The unsuccessful attempts to detect conventional virus particles in infected tobacco cells (Chapter VII) may have been due to a low titre of the agent, and to a highly specific location within the tissues. The agent may only exist with a protein coat at a certain stage of infection in certain tissues or organs, as has been reported for some plant viruses (Shikata and Maramorosch, 1966; Hartmann *et al.*, 1973). The possibility that TSA multiplies in xylem tissue of tobacco plants

should be reinvestigated, with special emphasis on a developmental study of infected plants.

Tobacco stunt is not considered to be associated with bacteria, rickettsia or Mollicutes-like organisms on the basis of sap transmission, inactivation by low concentrations of RNase, no remission of symptoms with antibiotics, and the absence of any microorganisms within the vascular tissues (Chapters V & VII). The aberrations in the vascular tissues were extensive (Chapter VII), but were not typical of infections with rickettsia and Mollicutes-like organisms (Hiruki and Dijkstra, 1973; Schneider, 1973).

The remote possibility that tobacco stunt is caused by tobacco necrosis virus is not excluded entirely, although in this study Red Kidney bean was used as a differential host for TNV. The unstable nature of TSA in sap, inactivation by low concentrations of RNase, and inability to demonstrate *in vitro* acquisition by zoospores suggest that TSA is distinct from TNV. Stunt agent is systemic in tobacco whereas TNV produces local infection on mechanically inoculated leaves and on *Olpidium*-inoculated roots. Hiruki (1975) also concluded that TSA differed greatly from TNV on the basis of host range, symptomatology, serology and cross protection tests. Further evidence concerning its relationship to TNV can only be provided when TSA has been successfully isolated and characterised.

Future attempts to isolate infectious TSA must involve the development of procedures for stabilising the agent during extraction. The selection of infected host material for TSA isolation may be important. Tomato may be a better host than tobacco because necrosis does not accompany TSA infection. If the stabilising effect of 4-PTC

on TSA infectivity is by inhibition of host polyphenol oxidases, then it should be best to isolate TSA from a host which does not contain high levels of polyphenols.

Mechanical transmission of TSA in sap has permitted a better understanding of this disease agent in the complex of agent, vector and host plant, and of the inter-relationships within this complex. Although TSA was extremely unstable in tobacco sap and infection rates varied in different experiments, the ability to transmit TSA in sap conferred advantages in using this system, rather than that of lettuce big-vein (Lin *et al.*, 1970), for an investigation of the internal and persistent transmission of a virus-like disease agent by a fungus vector.

EXPLANATION OF PLATES.

All plates have legends on the facing page to explain the material illustrated. The individual photographs making up each plate are marked A, B, C etc. At the end of each photomicrograph legend, symbols have been used to summarise the preparation and magnification of the subject matter. These symbols are sub-divided as follows: fixation / staining / approximate magnification / dimension of bar on photograph.

Explanation of symbols used:

Fixation:
- FG+O = fixation in 2% formaldehyde plus 2% glutaraldehyde followed by post fixation in 1%osmium tetroxide.
- K = fixation in 1% potassium permanganate.
- O = fixation in 1% osmium tetroxide.
- OV = fixation in vapour of 2% osmium tetroxide.
- B = fixation in boiling water.
- F = frozen in Freon 13 and freeze-dried.

Staining:
- UA+LC = staining in 2% aqueous uranyl acetate for 2 hours, followed by staining in 0.2% lead citrate for 4 minutes (Venable and Coggleshall, 1965).
- PTA = 2% aqueous phosphotungstate pH 7.0 as negative stain.
- AB = staining in 0.01% aniline blue in 1/15 M dibasic potassium phosphate pH 8.0 for 5 minutes.

The absence of a particular treatment is indicated by the * sign.

List of abbreviations and their meanings when used on the figures and in the legends:

a	axoneme	Lo	lomasome-like body
af	axonemal fibrils	PA	parenchyma
C	companion cell	PL	plastid
c	cyst	PP	P-protein
cc	cyst cytoplasm	r	rhizoplast
CH	chloroplast	S	sclerenchyma
cp	cyst plasmamembrane	SC	sieve cell
cw	cyst wall	SP	sieve plate
e	exit tube	ST	starch
eP	external phloem	t	thallus
f	flagellum	tp	thallus plasmamembrane
fc	fungal cytoplasm	tw	thallus wall
fm	fungal mitochondrion	V	vacuole
fn	fungal nucleus	W	wall
HC	host cytoplasm	X	xylem
HCW	host cell wall	XV	xylem vessel
HM	host mitochondrion	zb	zoospore body
HP	host plasmamembrane	zp	zoospore plasmamembrane
iP	internal phloem	zw	zoospore wall
k	kinetosome	☆	site of attachment of cyst to host cell wall.
L	lipid body		

PLATE 1. Incubators for culturing *Olpidium* on tobacco roots, and temperature treatment of stunt infected tobacco plants.

A. Pot incubator and cover for mass culture of *Olpidium* on tobacco roots.

B. Microincubators in wooden holder for culturing small populations of *Olpidium*.

C. Individual microincubator, consisting of a seedling support compartment (1) and a nutrient solution compartment (2), both of which are connected with a wool string wick (arrow).

D. Tobacco plants inoculated with zoospores of *Olpidium*/TSA and incubated at 17°C for 2 weeks, and at the following temperatures for a further 2 weeks: left, 17°C; centre, 25°C; right, 33°C.

E. Same tobacco plants as in D after further incubation at the following temperatures: left, 25°C; centre, 17°C; right, 17°C.

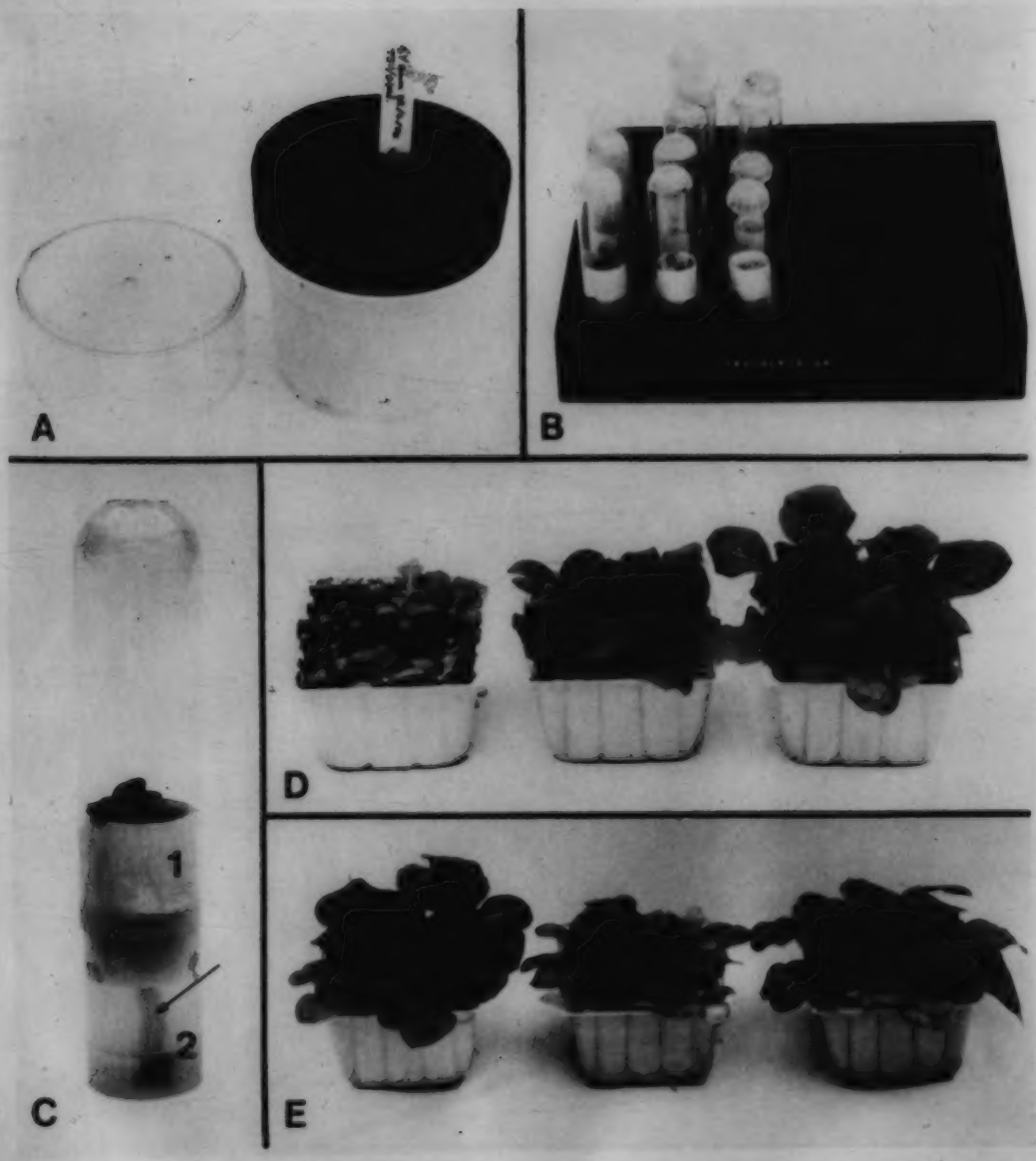

A
B
C
1
2
D
E

PLATE 2. Symptoms of tobacco stunt and tobacco necrosis.

A, B & C. Development of symptoms of stunt on tobacco leaves after inoculation with sap from tobacco showing stunt symptoms following inoculation with *Olpidium*/TSA.

A. Ring necrosis on inoculated leaf, 8 days after inoculation.

B. Systemic vein-clearing, 12 days after inoculation.

C. Systemic necrosis, 14 days after inoculation.

D. Tobacco plants 40 days after inoculation with *Olpidium* zoospores. Left, healthy: centre, *Olpidium:* right, *Olpidium*/TSA. Note the necrosis at soil level (arrows).

E. Local lesions on leaf of *Chenopodium amaranticolor* 14 days after inoculation with sap from tobacco leaves showing stunt symptoms following inoculation with *Olpidium*/TSA.

F. Local lesions on leaf of *C. amaranticolor* 7 days after inoculation with tobacco sap containing necrosis virus.

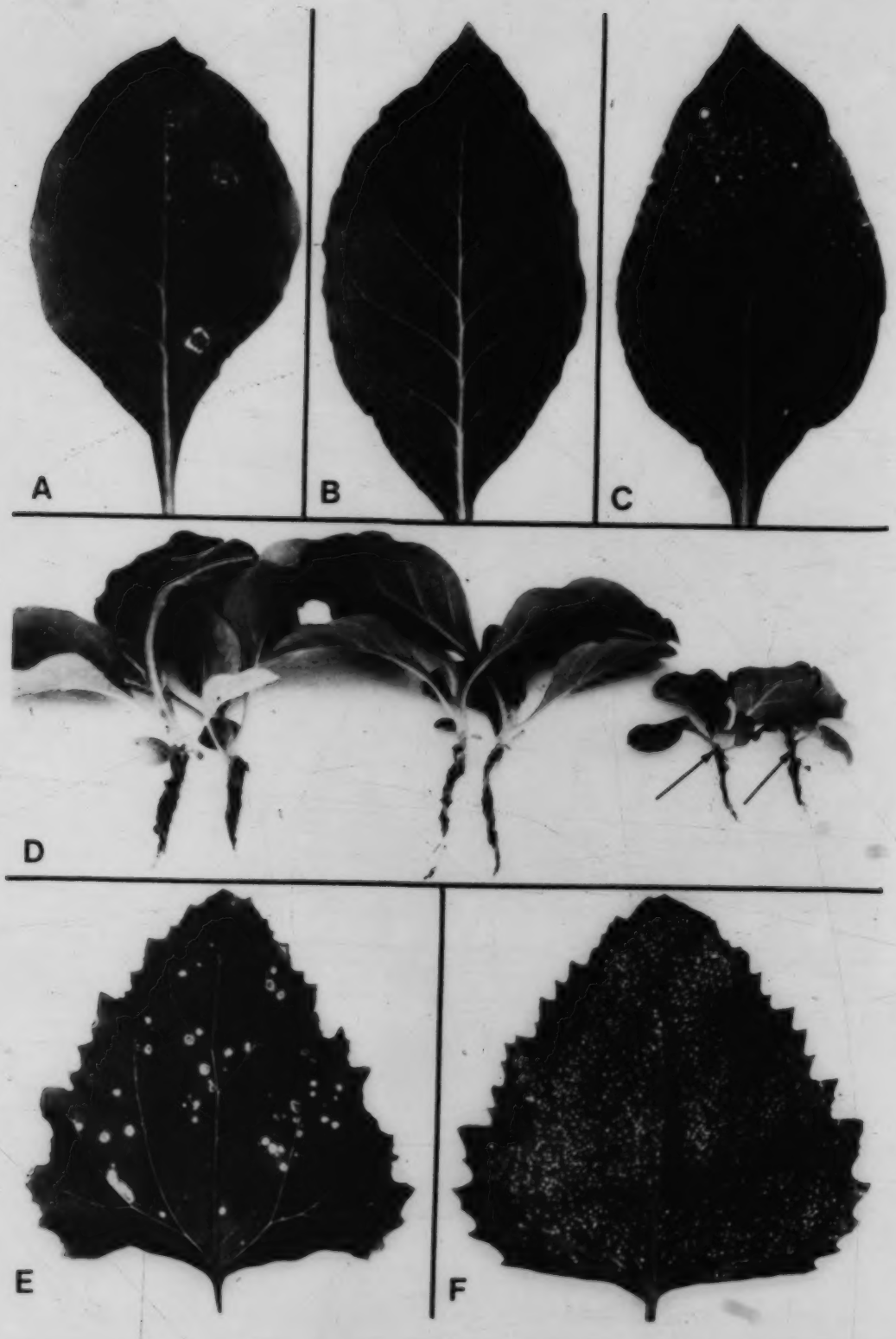

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PLATE 3. Symptoms of stunt disease in tobacco.

A. Tobacco plants 60 days after inoculation with *Olpidium* zoospores. Left, healthy: centre, *Olpidium*: right, *Olpidium*/TSA.

B. Tobacco plants 40 days after inoculation with zoospores of *Olpidium*/TSA. Note the systemic necrosis, rosetting and the distortion of developing leaves.

C. Tobacco plant grafted with tobacco scion showing stunt symptoms following inoculation with *Olpidium*/TSA. New growth from stock (arrows) shows stunting and systemic necrosis.

D. Tobacco plant 30 days after inoculation with sap from plant showing stunt symptoms following inoculation with *Olpidium*/TSA. Note local and systemic necrosis and stunting of the plant.

E. Tobacco plant 30 days after inoculation with sap as in D. Note the necrosis at soil level (arrows).

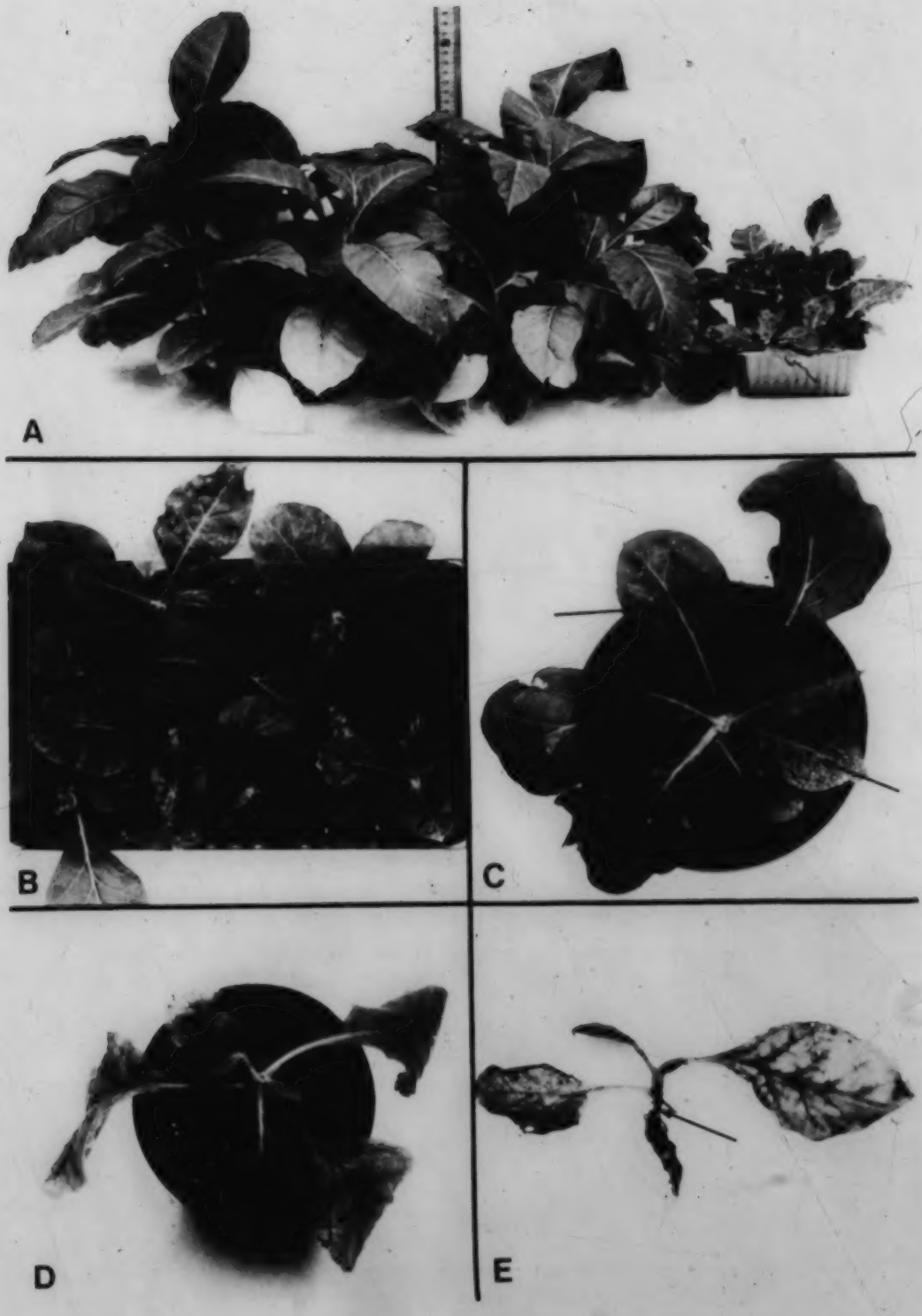
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PLATE 4. Light micrographs of stages in the life-cycle of the tobacco strain of *Olpidium*.

A. */*/3,200/4µm

B. */*/2,900/4µm

C. */*/750/15µm

D. */*/680/15µm

E. */*/330/30µm

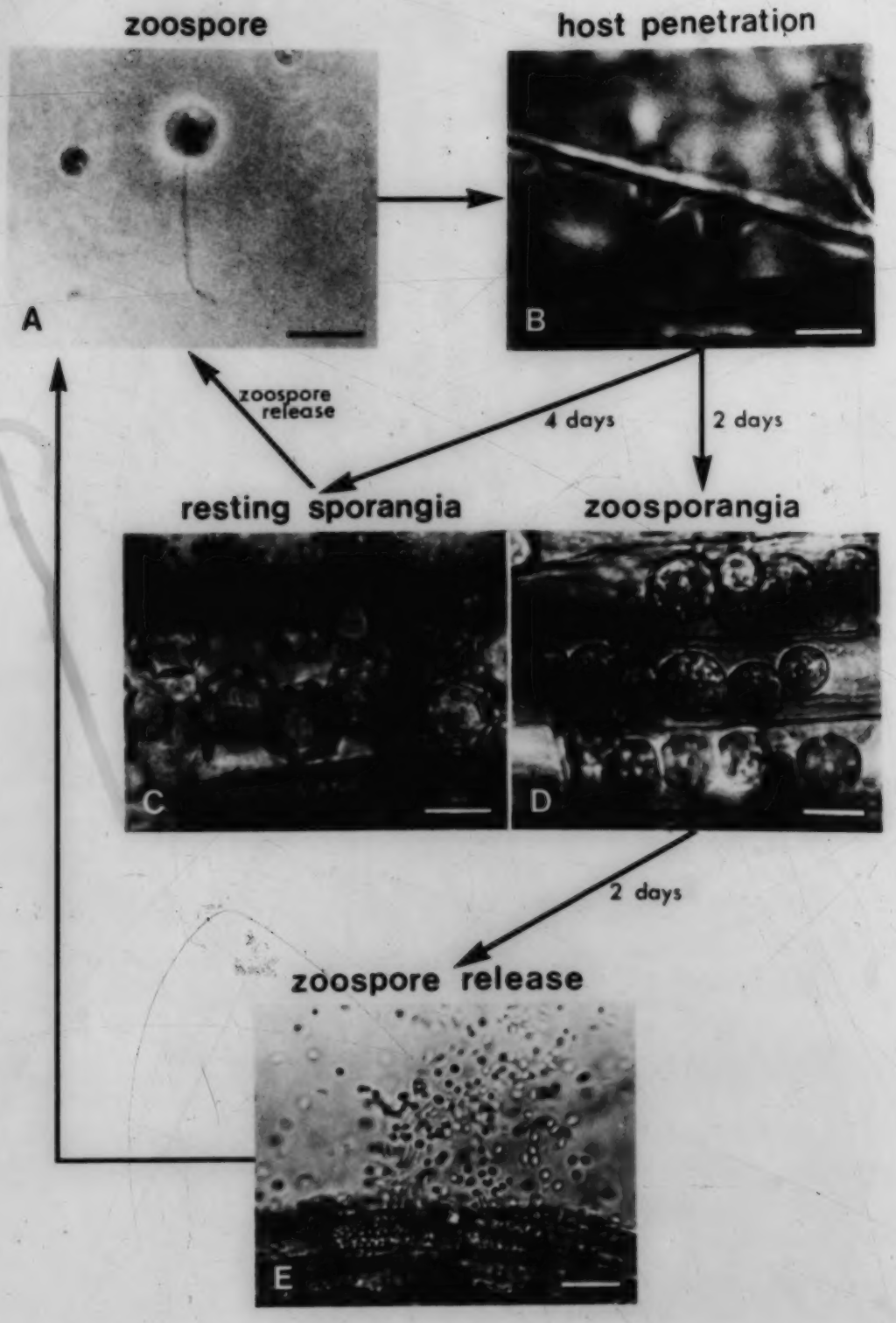

zoospore
host penetration
A
B
zoospore release
4 days
2 days
resting sporangia
zoosporangia
C
D
2 days
zoospore release
E

PLATE 5. Light micrographs of tobacco roots inoculated with *Olpidium* zoospores.

A, C & E. Roots stained with aniline blue and examined in visible light.

B, D & F. Same root tissues as in A, C & E respectively, examined in ultraviolet light.

A & B. Root incubated for 2 hours in zoospore suspension showing fluorescence in the zone of cell elongation. */AB/95/100µm.

C & D. Root incubated for 2 hours in zoospore suspension. Note the fluorescent areas (white arrows) in D which correspond with the positions of encysted zoospores (black arrows) in C. */AB/600/20µm.

E & F. Root incubated for 3 hours in zoospore suspension. Note the cell wall thickenings (white arrows) adjacent to the outlines of encysted zoospores (black arrows) in E, and the corresponding fluorescence (white arrows) in F. */AB/3,150/4µm.

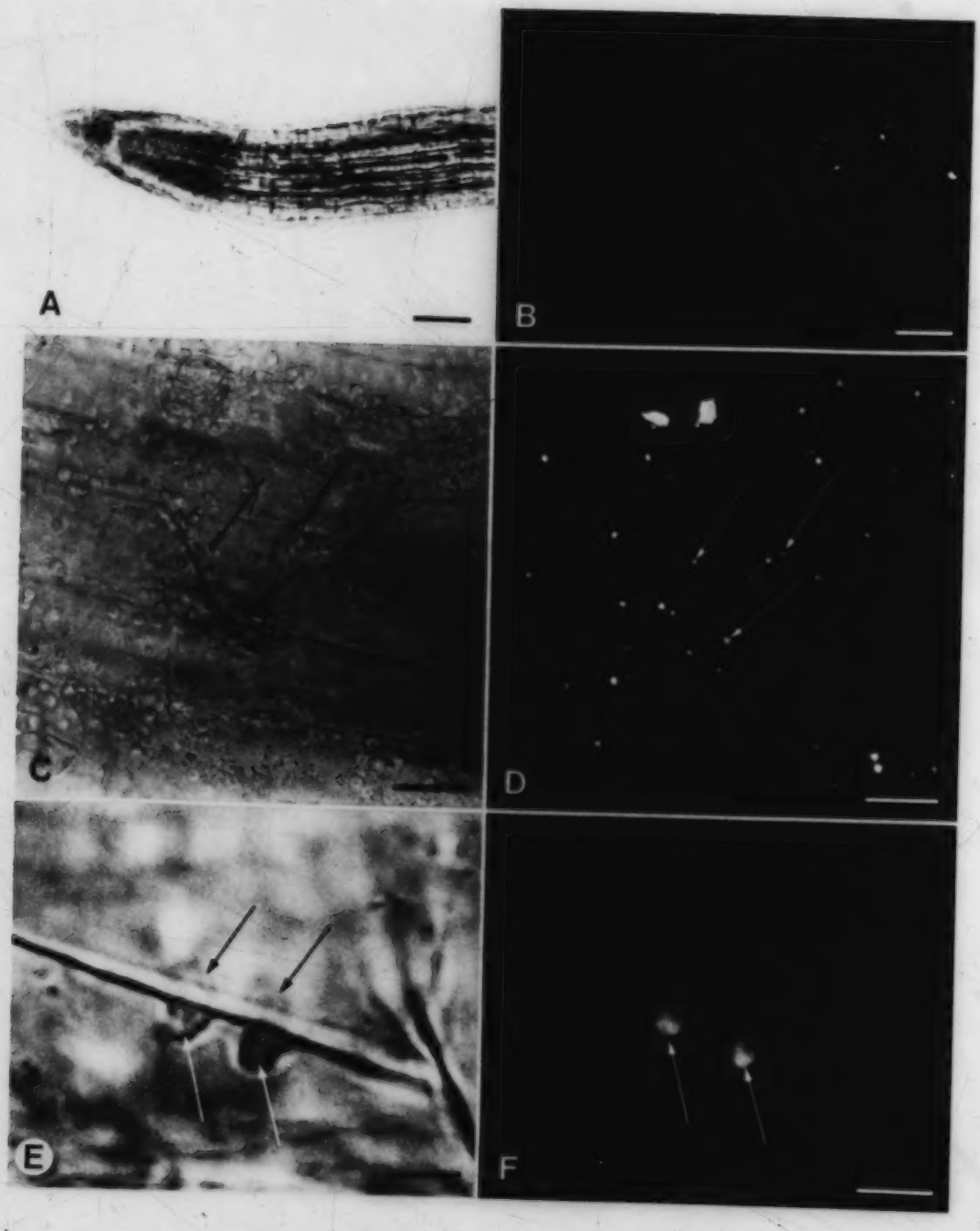

A
B
C
D
E
F

PLATE 6. Light micrographs of *Olpidium* thalli in tobacco root cells.

A. & B. Fungal thalli (black arrows) 12 hours after inoculation of root with zoospores. */AB/750/15µm.

A. Root examined in visible light.

B. Fluorescence micrograph of the same root tissue as examined in A. Note the absence of fluorescence in the corresponding positions of the thalli. Fluorescent areas (white arrows) in B correspond with the sites of penetration (white arrows) by zoospores in A.

C. Fungal thalli (arrows) 24 hours after inoculation of root with zoospores. */AB/950/10µm.

D. Zoosporangia (arrows) with exit tubes 48 hours after inoculation of root with zoospores. */AB/950/10µm.

E. Zoosporangia with exit tubes and resting sporangia (arrows), 96 hours after inoculation of root with zoospores. */AB/720/15µm.

F. Zoosporangium with many exit tubes, 96 hours after inoculation of root with zoospores. */AB/720/15µm.

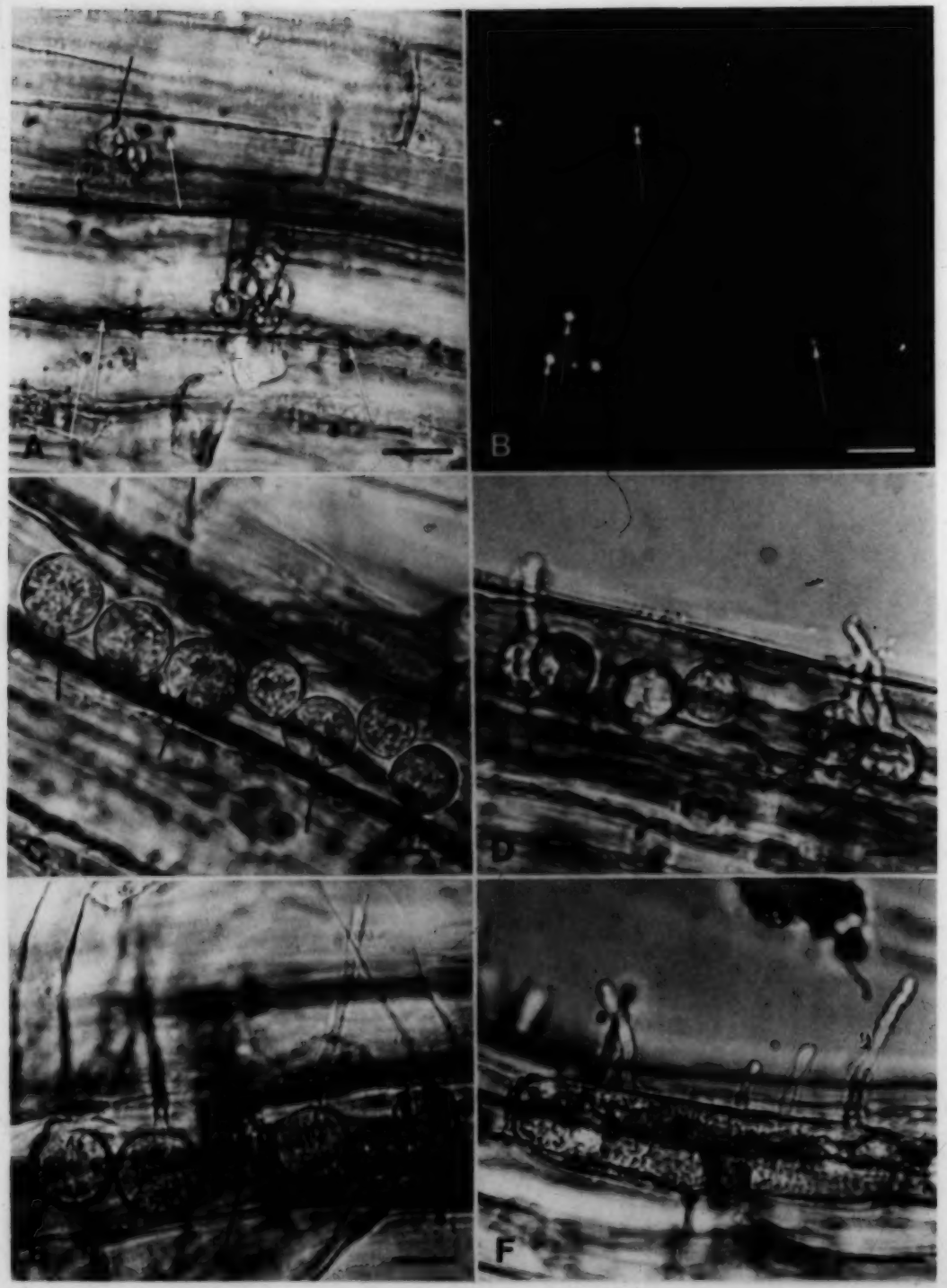

A
B
D
F

PLATE 7. Scanning electron micrographs of *Olpidium* zoospores: free and attached to tobacco roots.

A & B. Free *Olpidium*/TSA zoospores on Millipore filter.

A. OV/*/3,500/3μm.

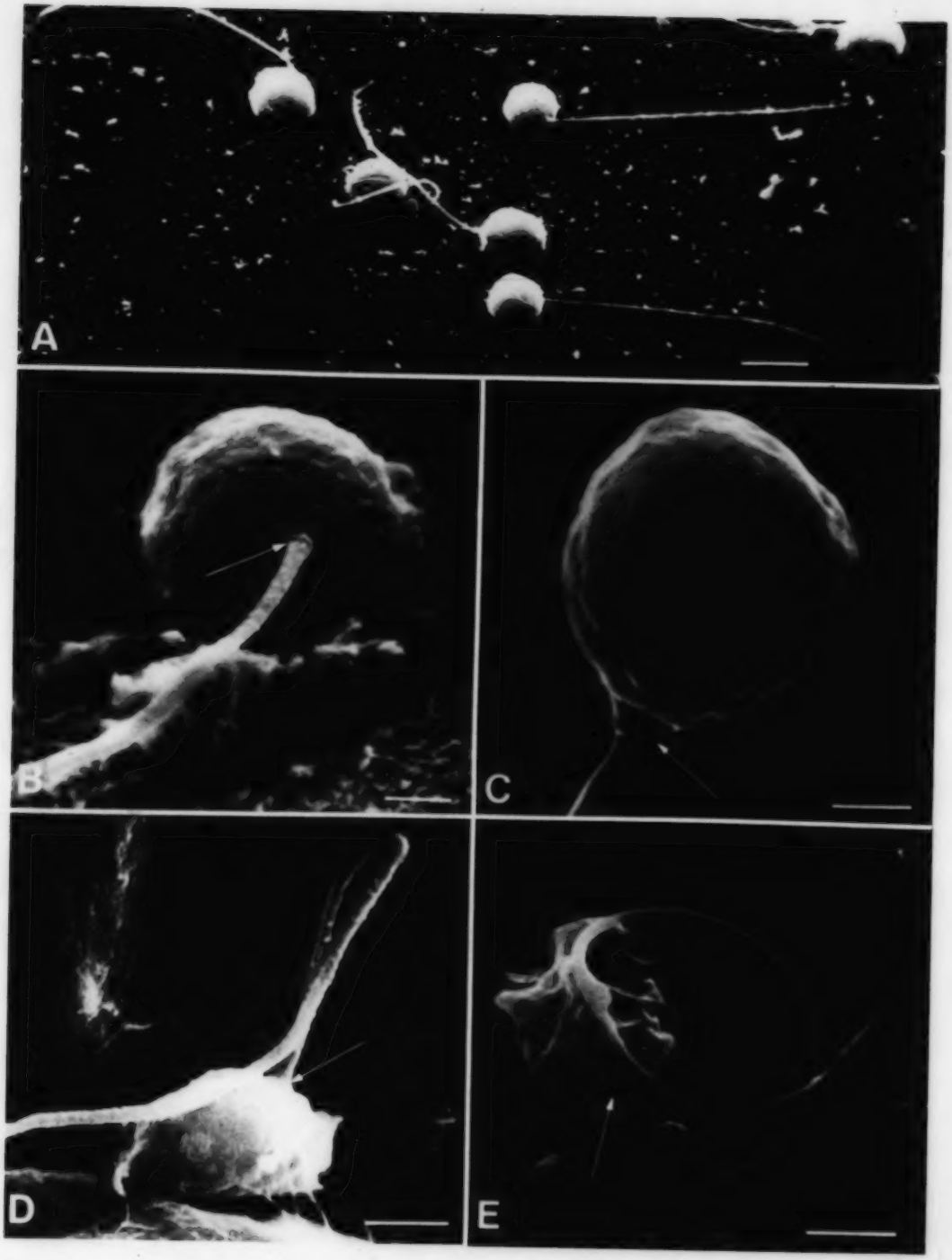
B. Note the ring-like swelling (arrow) where the flagellum joins the zoospore body. OV/*/20,000/0.5μm.

C. Free *Olpidium*/TSA zoospore on Nuclepore membrane. Note the ring-like swelling (arrow). OV/*/25,000/0.5μm.

D & E. Zoospores attached to tobacco roots and with the flagellum still present. Note the ring-like swelling (arrow)is still visible. Roots sampled after incubating for 10 minutes in zoospore suspension.

D. O/*/13,000/1μm.

E. FG+O/*/14,000/1μm.


A
B
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D
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PLATE 8. Scanning electron micrographs of *Olpidium* zoospores attached to tobacco roots.

A -E. Stages in retraction of the flagellum. Roots sampled after incubating for 10 minutes in zoospore suspension.

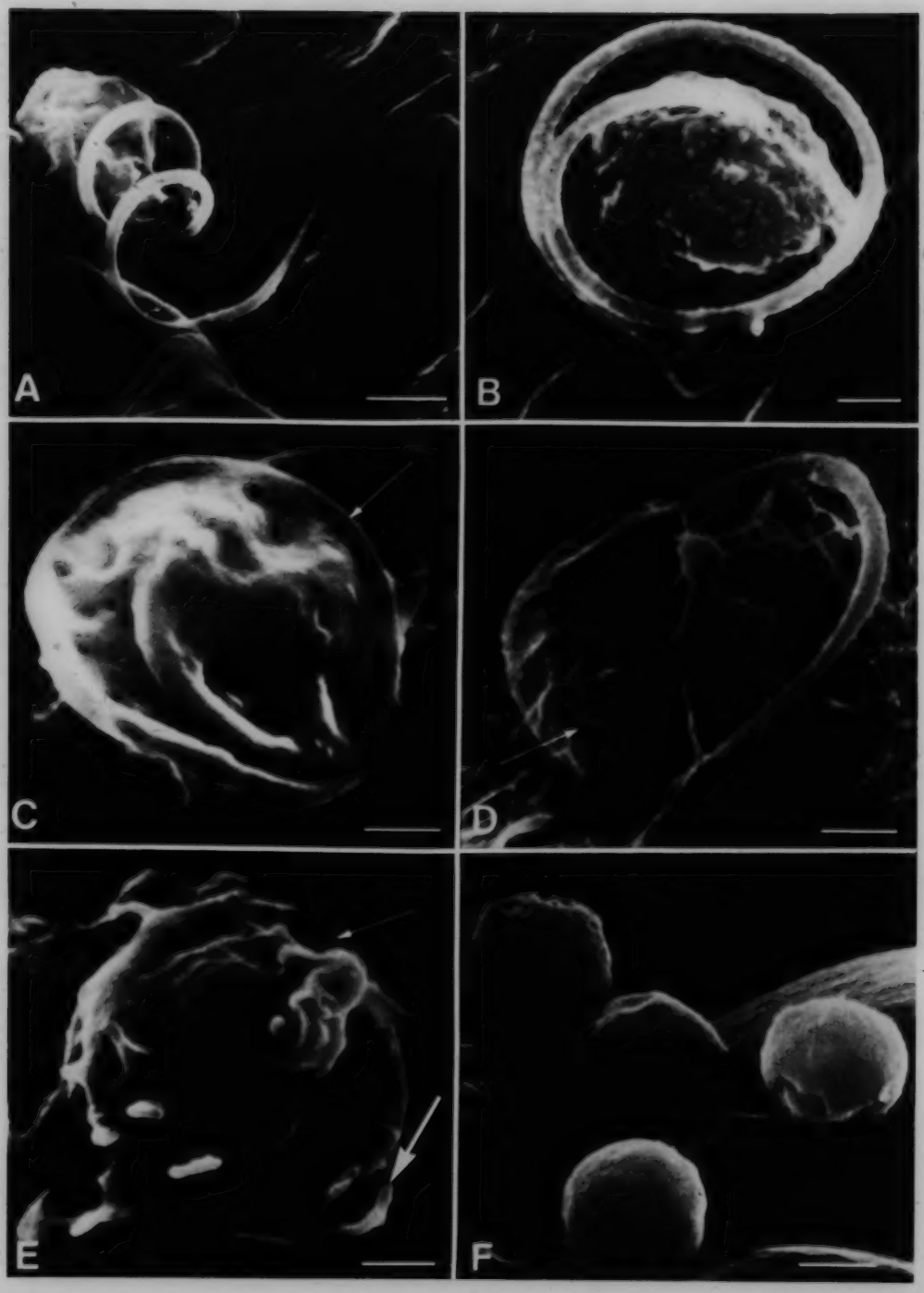
A. Zoospore with coiled flagellum. FG+O/*/12,500/1μm.

B. Flagellum coiled around the zoospore body. O/*/20,000/0.5μm.

C & D. Flagellum wrapped around the zoospore body. Apparent merging of the flagellum and body membranes (arrows) and ridge formation. FG+O/*/24,000/0.5μm.

E. Zoospore with flagellum (arrow) wrapped around its body and with the whiplash (large arrow) still free. FG+O/*/23,000/0.5μm.

F. Zoospores each with the flagellum completely retracted. Root sampled after incubating for 20 minutes in zoospore suspension. O/*/12,000/1μm.


A
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C
D
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F

PLATE 9. Scanning electron micrographs of *Olpidium* zoospores attached to tobacco roots.

A, B & C. Zoospores encysted on root in zone of cell elongation. Root sampled after incubating for 15 minutes in zoospore suspension.

A. FG+O/*/100/150μm.

B. Enlargement of area (arrow) in A. FG+O/*/500/30μm.

C. Enlargement of area (arrow) in B. FG+O/*/5,000/3μm.

D. Zoospores encysted on root hair. Enlargement of area (circle) in B. FG+O/*/6,000/2μm.

E & F. Freeze-fractured tobacco roots. Roots sampled 2 hours after inoculation with zoospores.

E. Fractured encysted zoospore. Cyst contains cytoplasm (cc) and large vacuole (arrow). FG+O/*/20,000/1μm.

F. Encysted zoospore (c) on root surface. Fracture through the host cell wall (HCW) showing thickening (arrow) adjacent to cyst. FG+O/*/24,000/0.5μm.

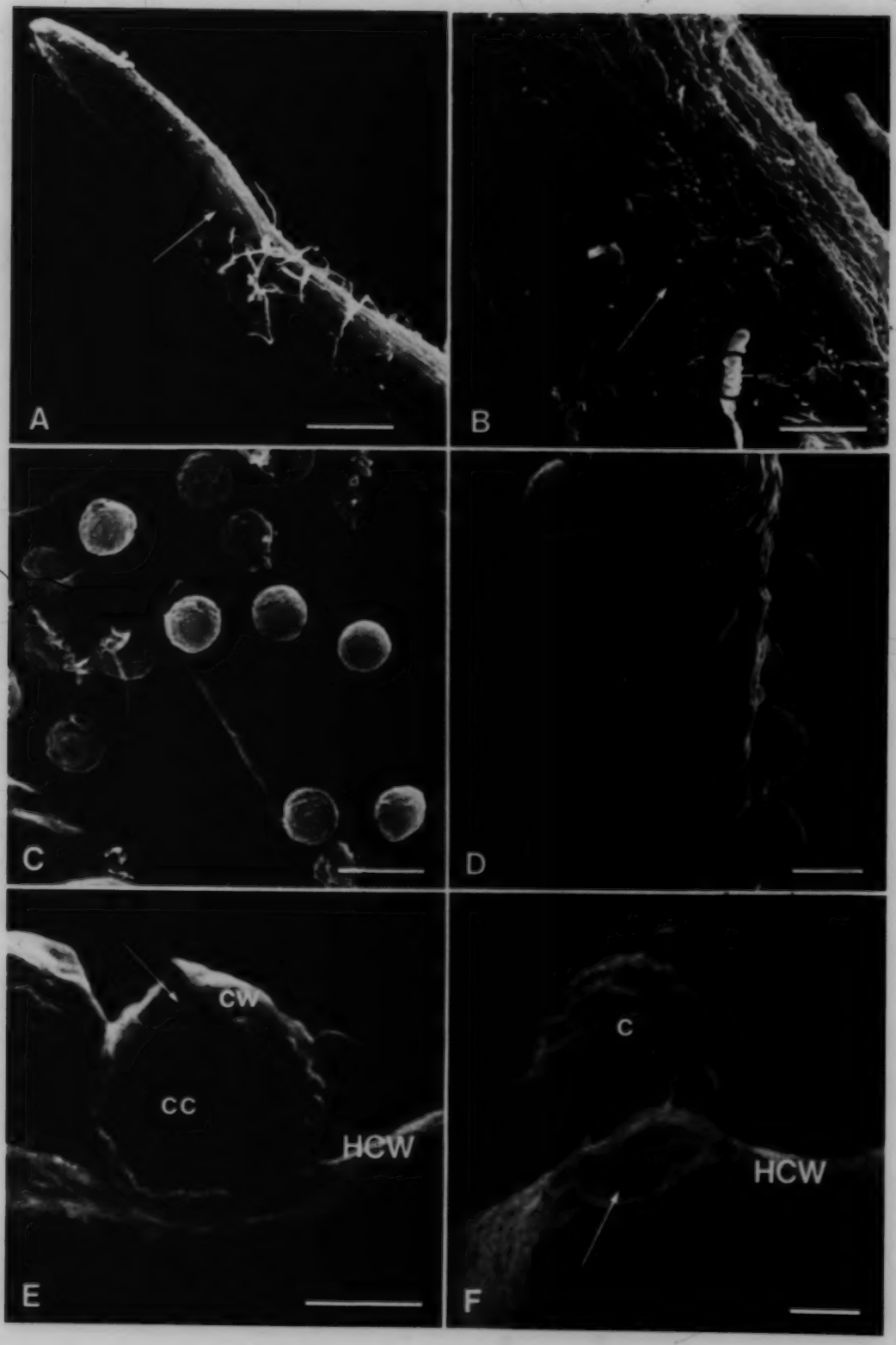

A
B
C
D
CW
CC
HCW
E
C
HCW
F

PLATE 10. Scanning electron micrographs of tobacco roots inoculated with *Olpidium* zoospores and freeze-fractured.

A. Fractured empty cyst showing the cyst wall (cw), 2 hours after inoculation. Note the thickening (arrow)of the host cell wall (HCW) adjacent to the cyst. FG+0/*/17,000/1μm.

B. Fractured cyst (c) and penetrating thallus (arrow), 3 hours after inoculation. No apparent thickening of the host cell wall (HCW). FG+0/*/14,000/1μm.

C. Two intact cysts (c) on fractured host cell wall (HCW), 4 hours after inoculation. Note the two penetrating thalli (arrows). FG+0/*/13,000/1μm.

D. Collapsed cysts (c) and adjacent cell wall thickenings (arrows), 24 hours after inoculation. FG+0/*/15,000/1μm.

E. Collapsed cyst (c) on root surface 24 hours after inoculation. Note the hole (arrow) in the host cell wall where penetration occurred. FG+0/*/26,000/0.5μm.

F. Intact and fractured (arrow) thalli (t) in an epidermal cell, 24 hours after inoculation. FG+0/*/7,200/2μm.

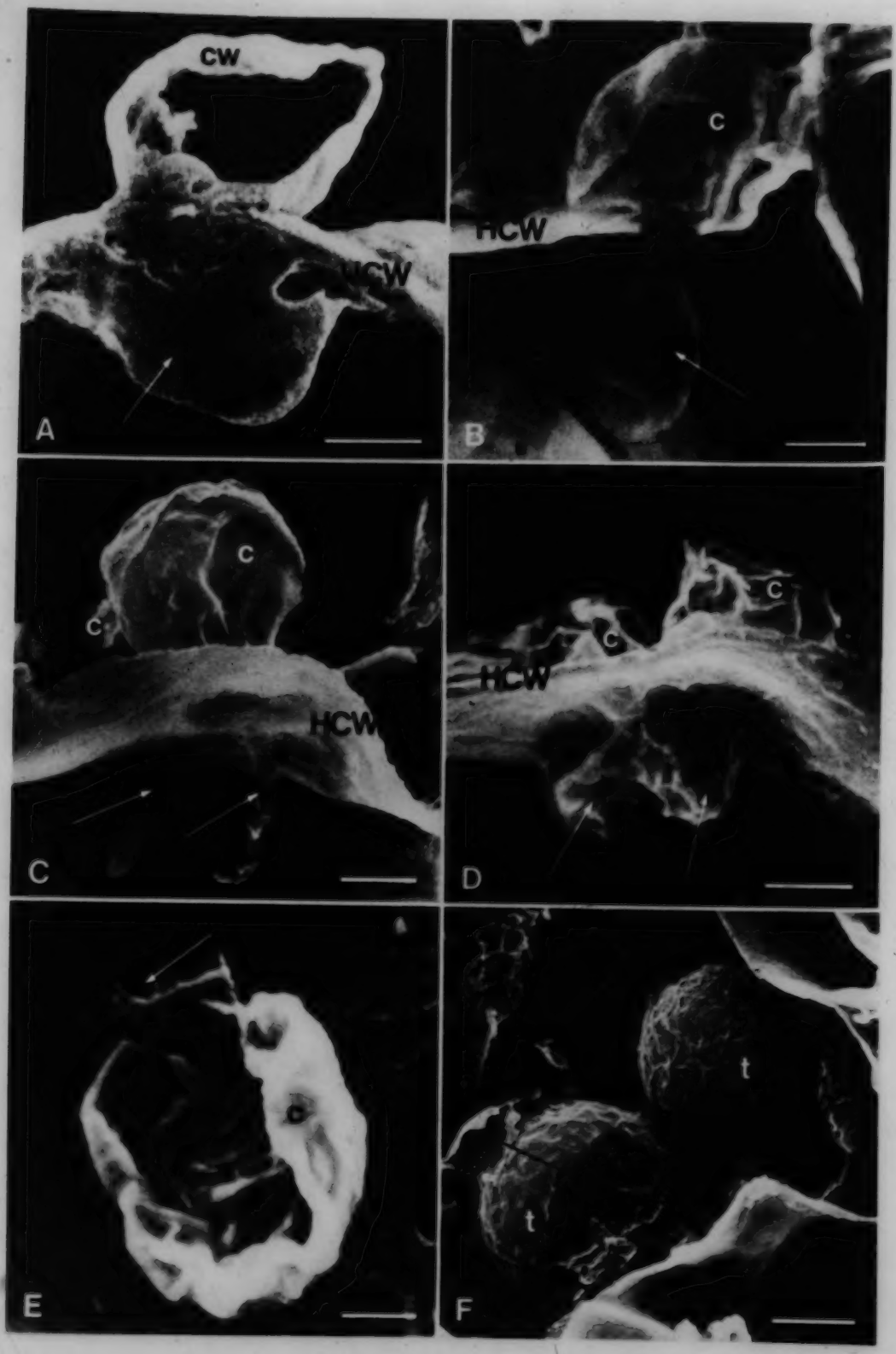

CW
HCW
A
c
HCW
B
c
c
HCW
C
c
c
HCW
D
E
t
t
F

PLATE 11. Scanning electron micrographs of tobacco roots inoculated with *Olpidium* zoospores and freeze-fractured.

A. Thalli (t) in an epidermal cell 48 hours after inoculation. FG+O/*/6,500/2μm.

B. Fractured thallus 48 hours after inoculation. Note that the thallus wall (arrow) separates the fungal cytoplasm (fc) from the surrounding host cytoplasm (HC). FG+O/*/5,5000/2μm.

C. Fractured thallus 48 hours after inoculation. Note the connection (arrow) between the thallus (t) and the root surface. FG+O/*/4,400/3μm.

D. Zoosporangium with exit tube (arrow), 72 hours after inoculation. FG+O/*/12,000/1μm.

E. Fractured zoosporangium 72 hours after inoculation. Note the presence of many vacuoles (v) in the fungal cytoplasm. FG+O/*/4,300/3μm.

F. Fractured mature zoosporangium containing zoospores (arrows), 96 hours after inoculation. FG+O/*/5,000/3μm.

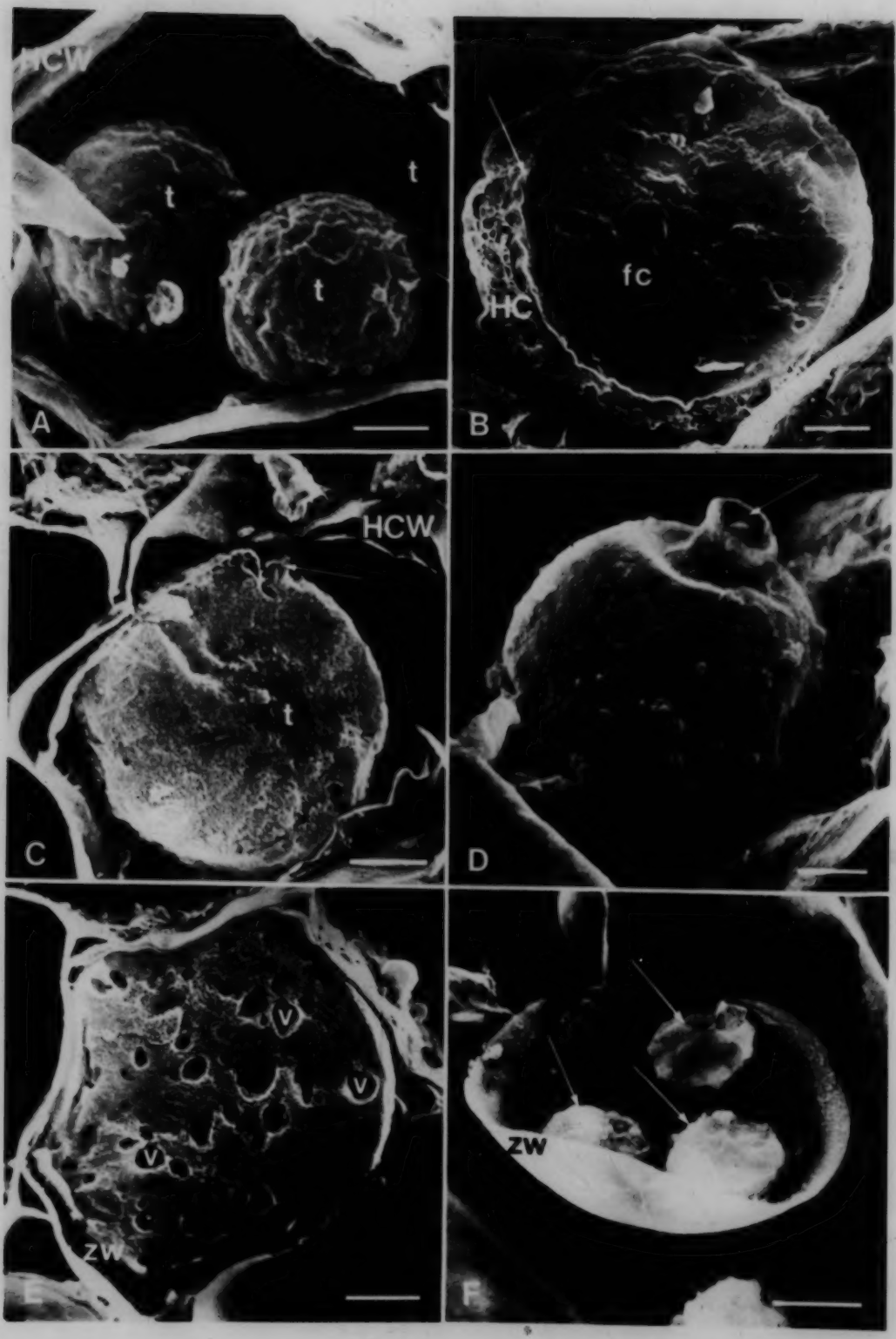

HCW
t
t
t
t
A
fc
HC
B
HCW
t
C
D
v
v
v
ZW
E
ZW
F

PLATE 12. Scanning electron micrographs of freeze-fractured tobacco roots containing *Olpidium* zoosporangia and resting sporangia.

A. Zoosporangium with exit tube (arrow), 96 hours after inoculation of root with zoospores. FG+0/*/4,200/3μm.

B. Young resting sporangium, 96 hours after inoculation of root with zoospores. Ridges (arrows) visible on the surface. FG+0/*/6,200/2μm.

C. Resting sporangia in epidermal cell, 96 hours after inoculation of root with zoospores. Ridges are pronounced. FG+0/*/5,000/3μm.

D. Resting sporangia in epidermal cells, 120 hours after inoculation of the root with zoospores. FG+0/*/3,400/3μm.

E. Longitudinal fracture of root showing resting sporangia in cells of the cortex. F/*/800/15μm.

F. Transverse fracture of root showing resting sporangia in epidermal cells and cells of the cortex. Note the presence also of a zoosporangium (arrow) with a long exit tube. F/*/1,000/10μm.

HCW
A
B
HCW
C
D
HCW
E
F

PLATE 13. Scanning electron micrographs of *Olpidium* resting sporangia in freeze-fractured tobacco roots.

A. Resting sporangium with ridges making up five and six-sided facets. FG+0/*/5,500/2μm.

B. Fractured resting sporangium. Note the thick wall (W) surrounding the fungal cytoplasm (fc) which has a sculptured surface with a 'raspberry-like' appearance. FG+0/*/9,000/2μm.

C. Fractured resting sporangium with sculpture of rod-like depressions on the surface of the cytoplasm (fc). FG+0/*/4,500/3μm.

D. Enlargement of C to show the layers of the wall (W) and the rod-like depressions on the surface of the cytoplasm (fc). FG+0/*/9,000/2μm.

E. Paired fractured surface of resting sporangium in C. Note the rod-like raised areas (arrow). FG+0/*/18,000/1μm.

F. Enlargement of E to show the rod-like raised areas. FG+0/*/4,500/3μm.

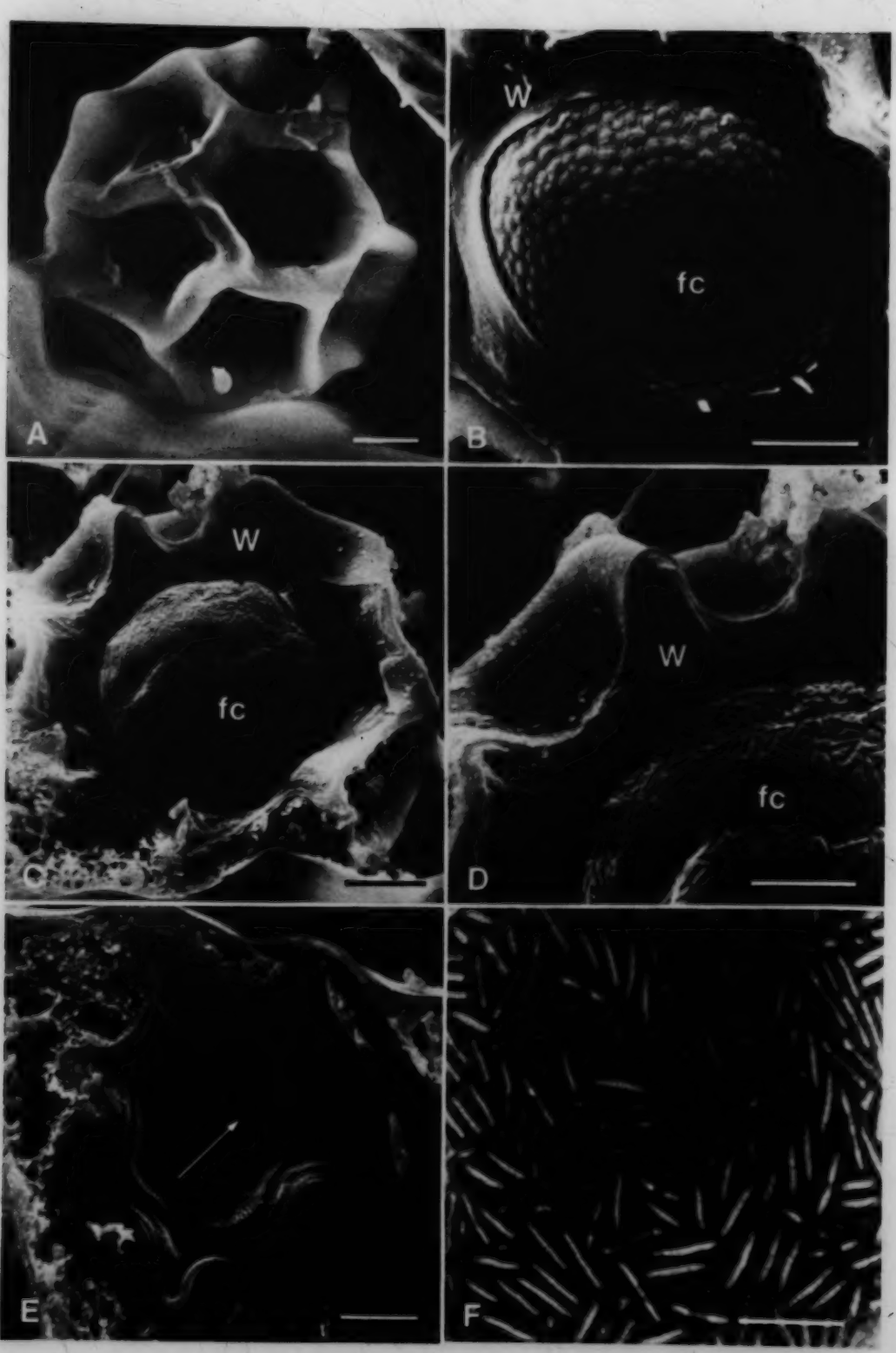

A
B
W
fc
C
W
fc
D
W
fc
E
F

PLATE 14. Transmission electron micrographs of zoospores of *Olpidium*/TSA.

A. Intact zoospore with plasmamembrane (arrows) around the body (zb) and flagellum (f). OV/*/7,500/2μm.

B. Longitudinal section through zoospore. Note the slight swelling (arrow) where the flagellum joins the body. FG+O/UA+LC/29,500/0.5μm.

C. Longitudinal section through zoospore body and part of the flagellum showing the plasmamembrane (zp) continuous around the body and flagellum. Note that the central fibrils (arrow) of the flagellum terminate at the kinetosome (k). FG+O/UA+LC/56,000/0.2μm.

D. Transverse section of flagellum showing 9+2 arrangement of axonemal fibrils surrounded by plasmamembrane. FG+O/UA+LC/89,000/0.1μm.

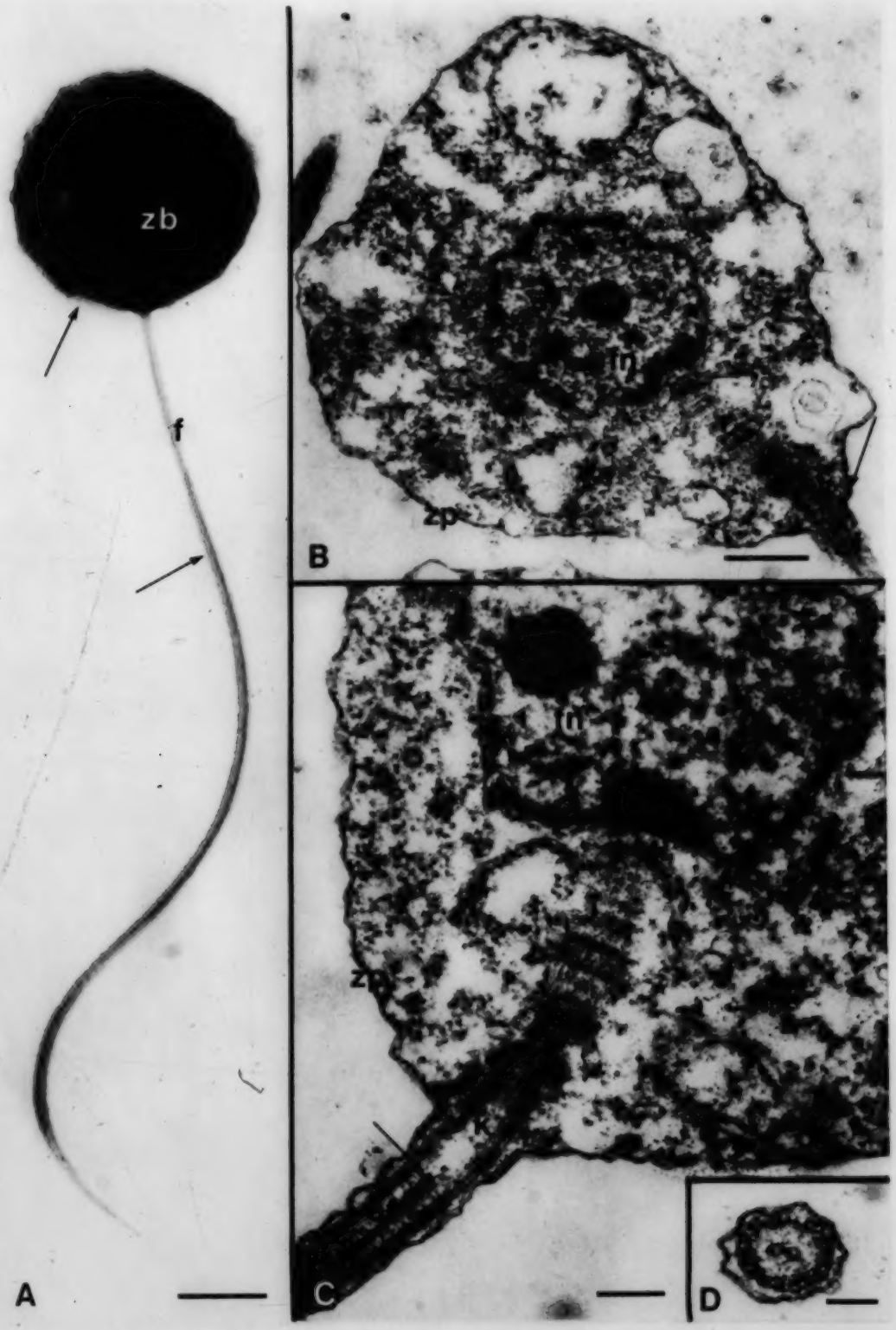

zb
f
A
n
zp
B
n
zp
C
D

PLATE 15. Transmission electron micrographs of *Olpidium* zoospores attached to tobacco roots.

A - D. Sections of zoospores on roots sampled after incubating for 10 minutes in zoospore suspension.

A. Zoospore undergoing retraction of the flagellum.
FG+O/UA+LC/46,000/0.3μm.

B. Enlargement of transverse section of axonemal fibrils (af) in A. Note that the 2 central fibrils (arrow) are present in this section and that the outer fibrils are surrounded by zoospore cytoplasm. The flagellum membrane is absent around the axonemal fibrils.
FG+O/UA+LC/95,700/0.1μm.

C & D. Zoospores each with the flagellum retracted. Cyst wall not present around the zoospore bodies, which are surrounded by only the plasmamembrane (zp). Axonemal fibrils (arrows) are present in the zoospore cytoplasm in transverse section in C, and in oblique section in D. The fibrils are not surrounded by a membrane.
FG+O/UA+LC/32,000/0.5μm.

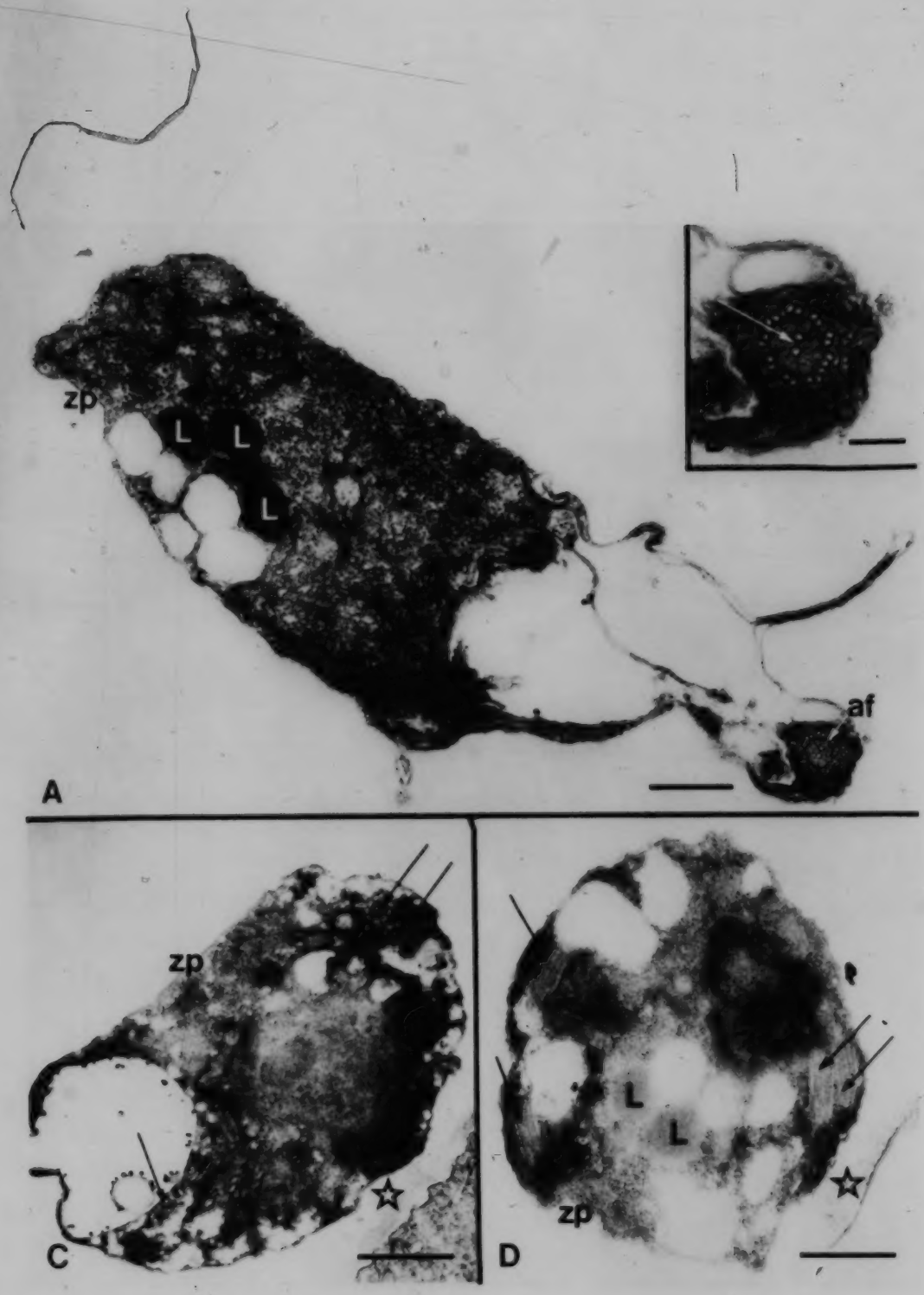

zp
L
L
L
af
A
zp
C
zp
L
L
D

PLATE 16. Transmission electron micrographs of *Olpidium*/TSA zoospore attached to tobacco root.

A & B. Sections through a zoospore body with the axonemal fibrils (af) present in the cytoplasm. Root sampled after incubating for 15 minutes in zoospore suspension. Note the absence of the flagellum membrane around the axonemal fibrils, and the thin layer of cyst wall material (arrows) deposited exterior to the body plasmamembrane (cp). FG+O/UA+LC/33,600/0.5µm.

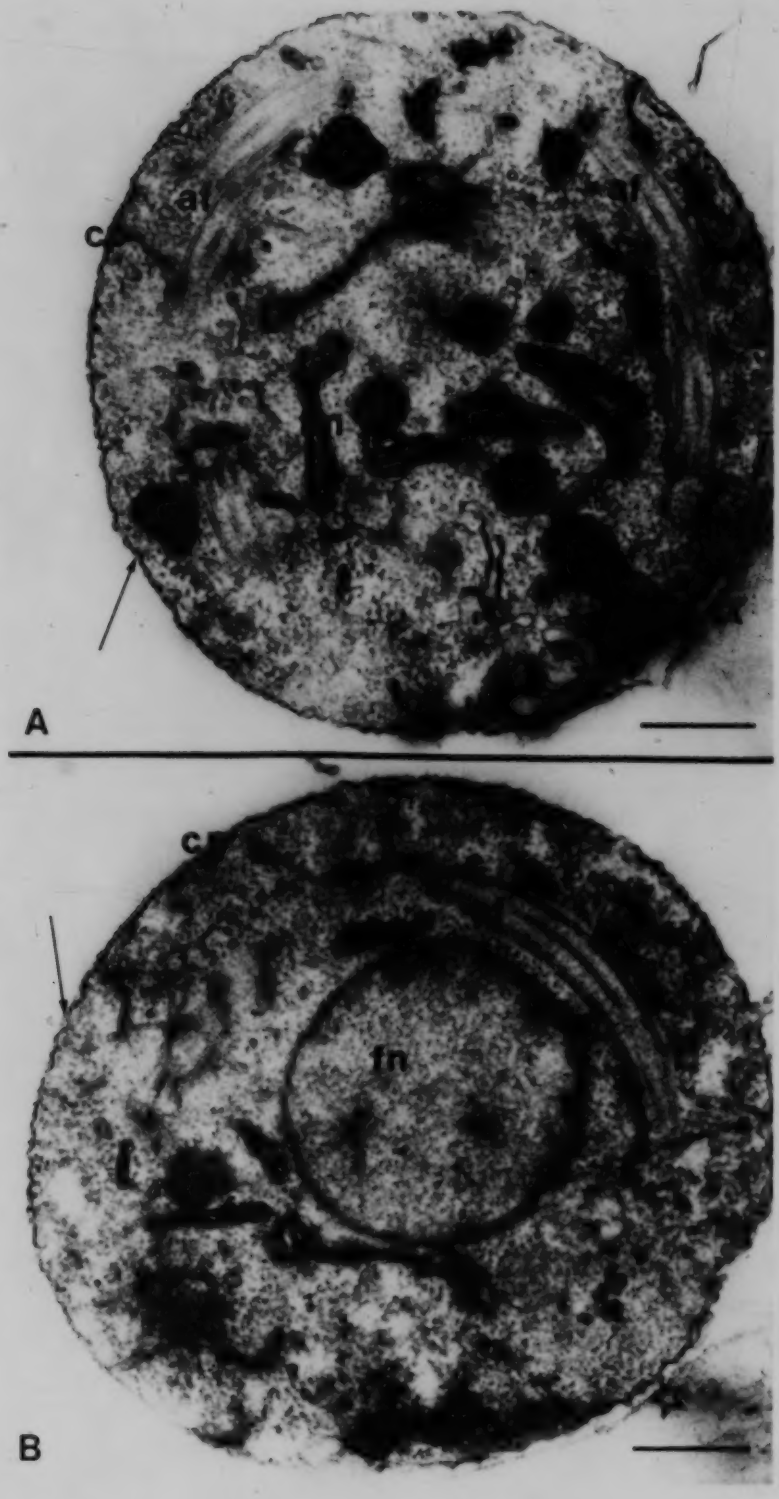
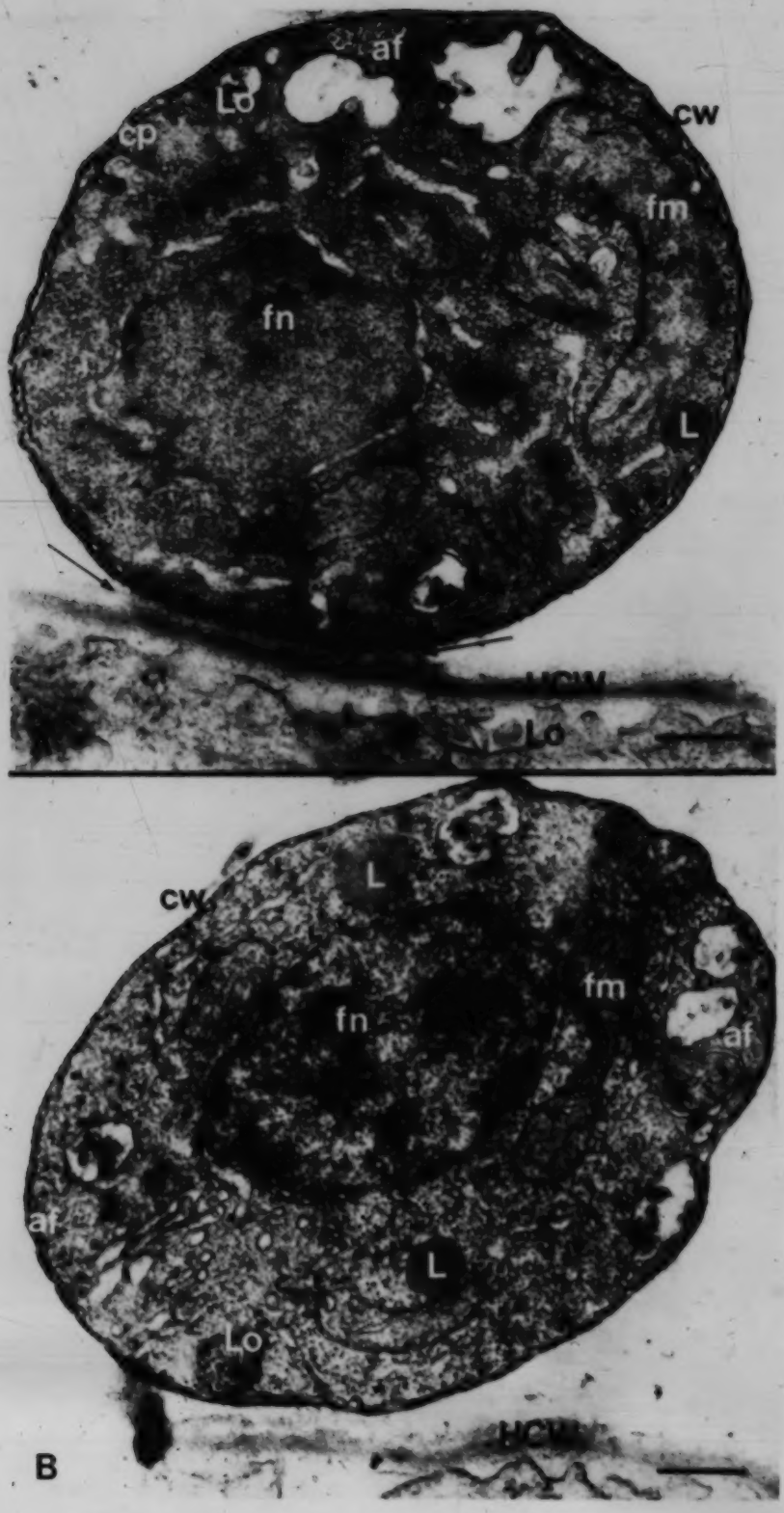

A
B
fn

PLATE 17. Transmission electron micrographs of *Olpidium* zoospores encysted on tobacco roots.

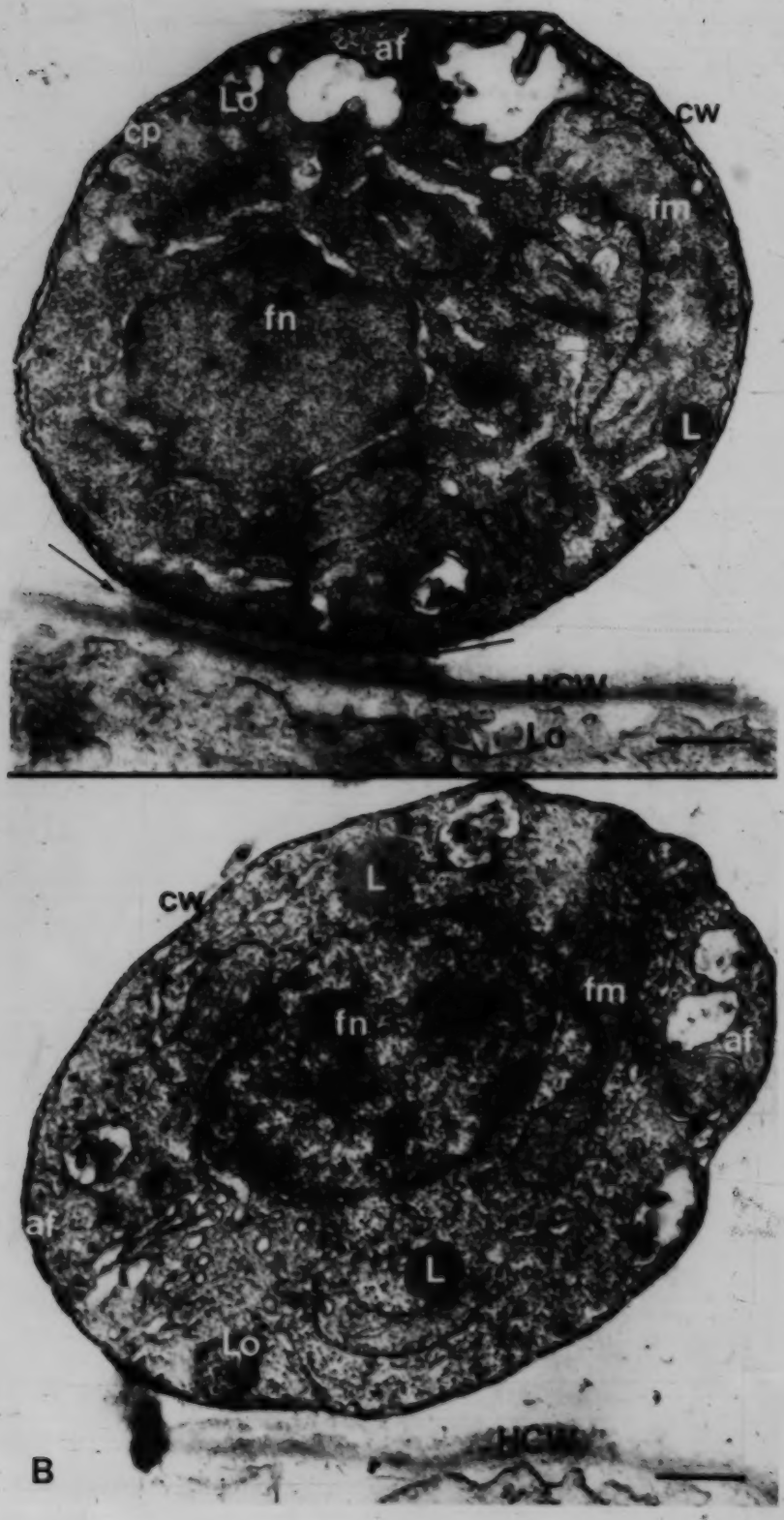
A & B. Roots sampled one hour after inoculation. Note the cyst wall (cw) deposited exterior to the plasmamembrane. Axonemal fibrils (af) are present in transverse section but not in the regular 9+2 arrangement.

A. Section of encysted *Olpidium* zoospore. Note the electron dense material (arrows) between the cyst wall and the host cell wall (HCW). Lomasome-like bodies (Lo) are present between the host plasmamembrane and cell wall adjacent to the encysted zoospore. FG+O/UA+LC/45,000/0.3µm.

B. Section of encysted *Olpidium*/TSA zoospore. FG+O/UA+LC/44,000/0.3µm.



B



B

PLATE 18. Transmission electron micrographs of encysted *Olpidium* zoospores penetrating tobacco root cells.

A, B & C. Roots sampled 3 hours after inoculation.

A. Oblique section through an encysted *Olpidium* zoospore. Note the presence of a large vacuole (V) distal to the site of attachment to the host cell wall (HCW), and the presence of a lomasome-like body (Lo) near the site of attachment. FG+O/UA+LC/46,800/0.3μm.

B. Oblique section through the penetration channel (arrow) and thickening of the host cell wall (HCW). Lomasome-like bodies are present in the cyst and the penetration channel. Material has been deposited between the host cell plasmamembrane (HP) and the cell wall. FG+O/UA+LC/36,000/o.3μm.

C. Section through penetration channel (arrow) and thickening of host cell wall (HCW). Most of the fungal cytoplasm (fc), including the nucleus (fn), is present inside the host cell, and separated from the host cytoplasm by only the thallus plasmamembrane (tp). Some fungal cytoplasm and lomasome-like bodies (Lo) are still present in the cyst (c). FG+O/UA+LC/40,300/0.3μm.

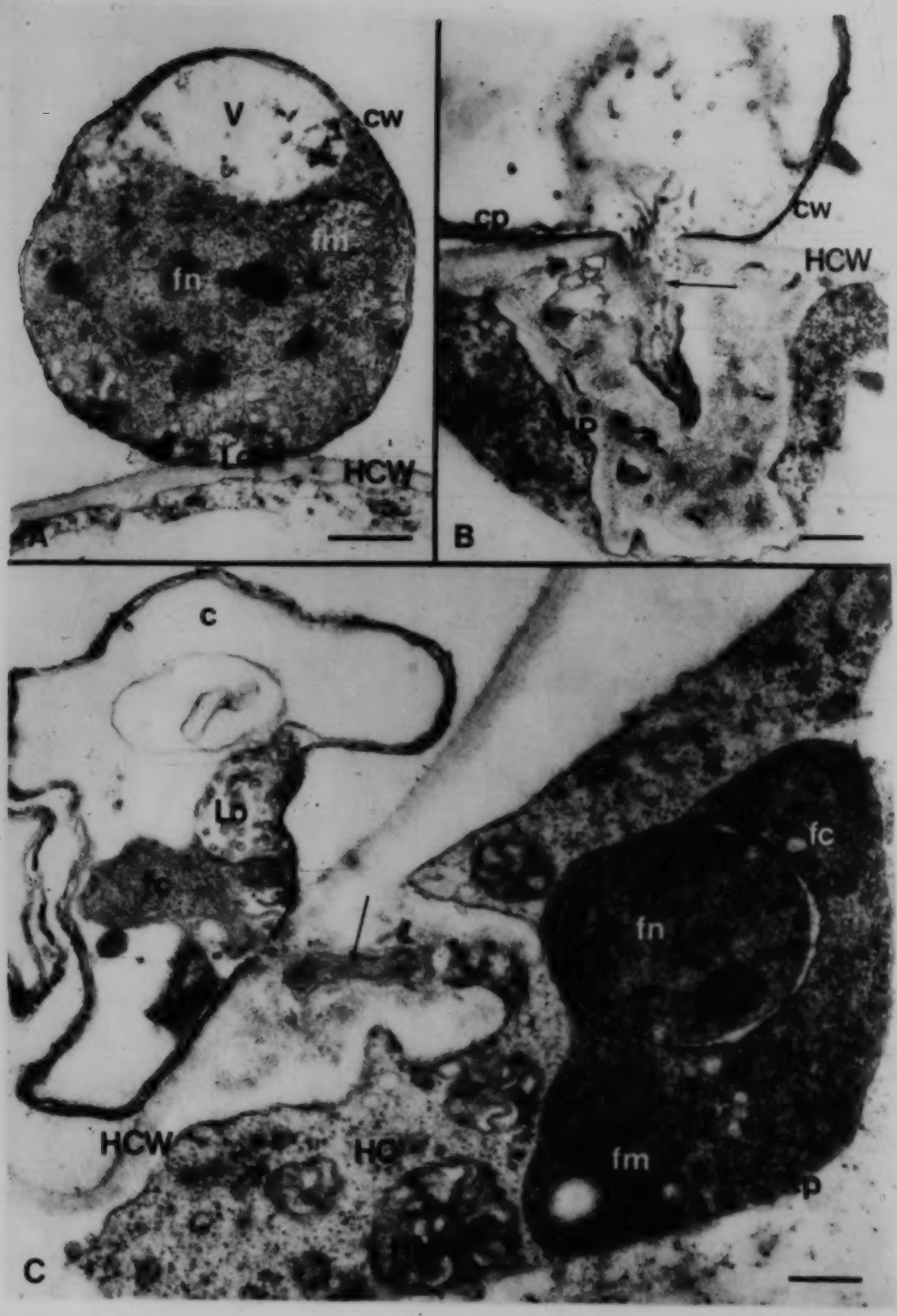

V
CW
fm
fn
HCW
A
CP
CW
HCW
B
c
Ld
fc
fn
HCW
HC
fm
C

PLATE 19. Transmission electron micrographs of *Olpidium* thalli in tobacco root cells.

A. Thallus (t) separated from host cytoplasm (HC) by only the thallus plasmamembrane (tp). Root sampled 12 hours after inoculation with zoospores. FG+O/UA+LC/36,000/0.3μm.

B. Thallus with thin wall present (arrow). Root sampled 36 hours after inoculation with zoospores. FG+O/UA+LC/7,400/2μm.

C. Thallus with definite wall (tw), which is deposited exterior to the thallus plasmamembrane. Root sampled 36 hours after inoculation with zoospores. FG+O/UA+LC/13,000/1μm.

D. Thallus with wall (tw), and many vesicles (arrows) present in the cytoplasm. Root sampled 48 hours after inoculation with zoospores. FG+O/UA+LC/37,300/0.3μm.

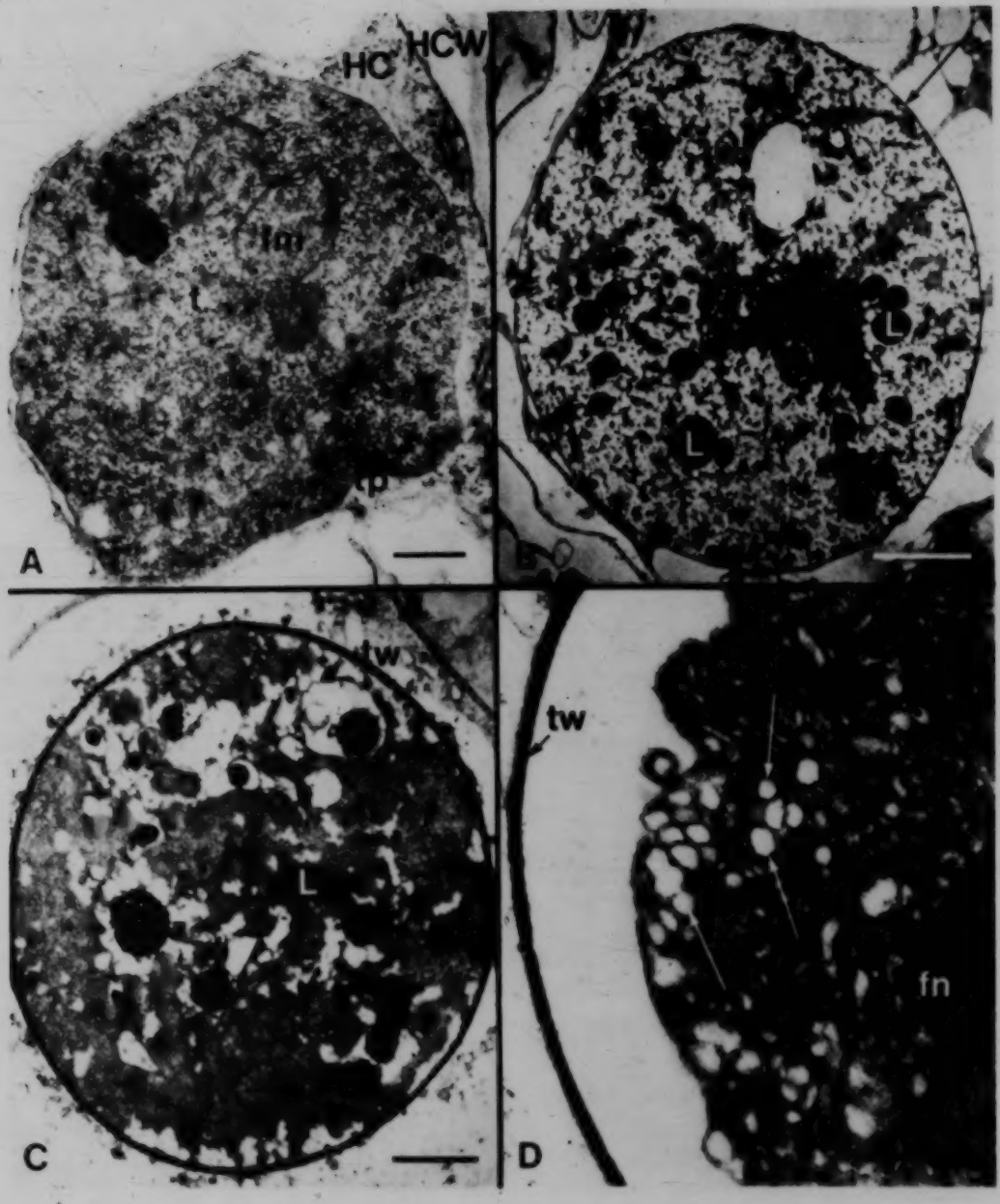

HC
HCW
fn
L
A
L
L
tw
L
C
tw
fn
D

PLATE 20. Transmission electron micrographs of *Olpidium* zoosporangia in tobacco roots.

A. Part of an *Olpidium*/TSA zoosporangium. The wall (zw) appears to have two layers of different electron density. The cytoplasm is partly differentiated to form zoospores, and oblique sections of axonemes (a) are visible. FG+O/UA+LC/27,000/0.5µm.

B. Mature zoosporangium of *Olpidium*/TSA with a plugged exit tube (e). The cytoplasm has differentiated to form zoospores. FG+O/UA+LC/4,2oo/0.3µm.

C. Section through a zoospore in a mature *Olpidium* zoosporangium. Note the transverse sections of axonemes (arrows). The zoospore body is surrounded by a single membrane (zp) and the cytoplasm varies in density. FG+O/UA+LC/27,000/0.4µm.

D. Section through a zoospore in a mature *Olpidium*/TSA zoosporangium. The relationship between the nucleus (fn), rhizoplast (r), kinetosome (k) and axoneme (a) is visible. FG+O/UA+LC/29,700/0.4µm.

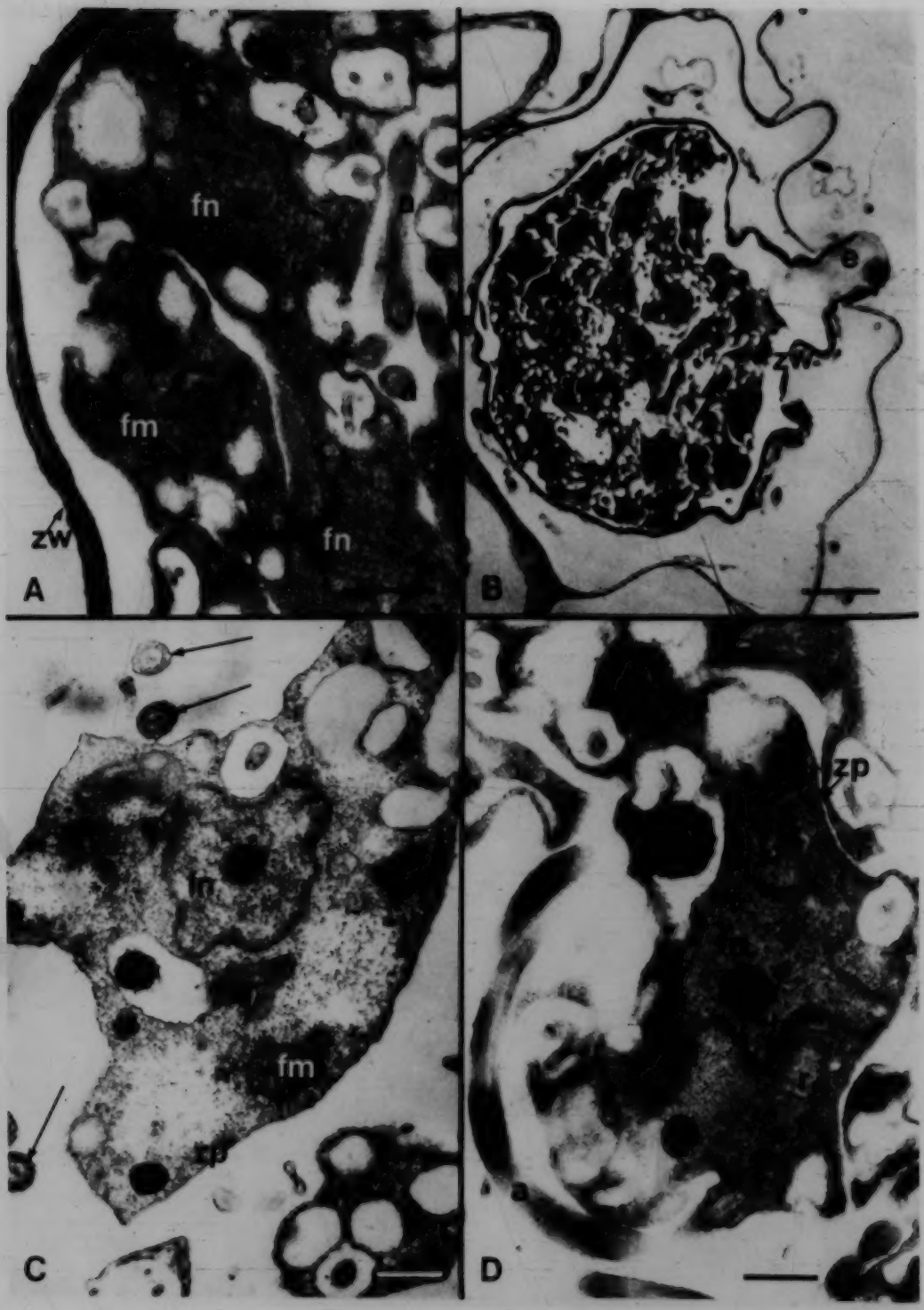

fn
fm
zw
fn
A
B
fn
fm
C
zp
D

PLATE 21. Transmission electron micrographs of *Olpidium* resting sporangia in tobacco roots.

A & B. Young resting sporangia with thick walls (W). The cytoplasm is dense, but organelles are still visible.

A. Many mitochondria (fm) are present in the fungal cytoplasm (fc). FG+O/UA+LC/12,500/1μm.

B. Note that the host cell plasmamembrane (arrows) is detached from the host cell wall (HCW). FG+O/UA+LC/15,500/1μm.

C. Developing resting sporangium of *Olpidium*/TSA with thick, undulating wall (W). The cytoplasm is extremely dense, and contains numerous large bodies. K/UA+LC/7,100/2μm.

D. Enlargement of wall in C to show layers of wall and fibrous structures (arrows). K/UA+LC/36,000/0.3μm.

E. Resting sporangium with fully developed wall (W), showing the characteristic stellate outline. Note the dense cytoplasm (fc) and numerous lipid bodies at the periphery of the cytoplasm. FG+O/UA+LC/4,800/3μm.

F. Enlargement of wall in D to show the different layers present (1, 2 & 3). FG+O/UA+LC/38,000/0.3μm.

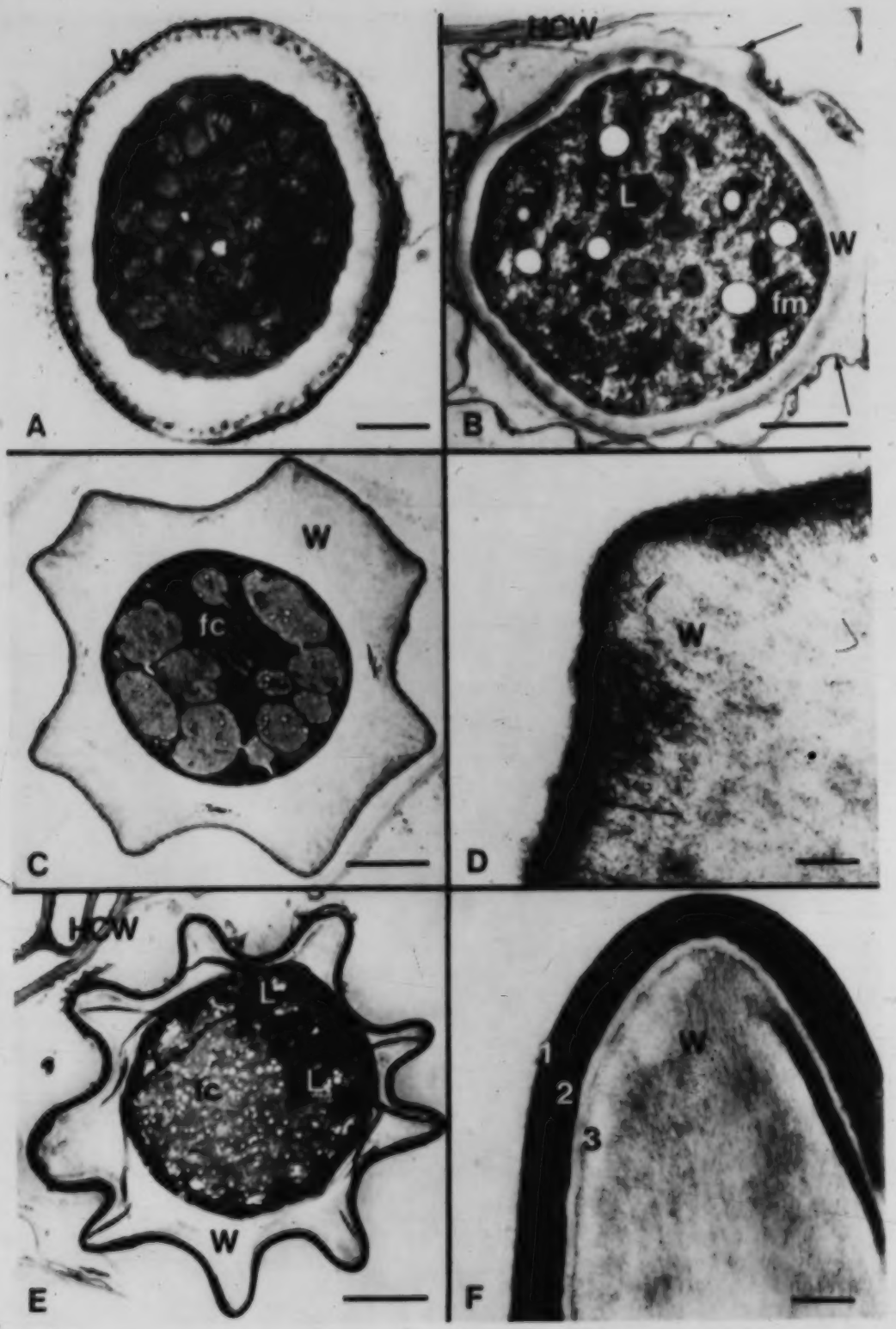

HCW
L
W
fm
A
B
fc
C
D
E
F
1
2
3

PLATE 22. Light and fluorescence micrographs of tobacco stems.

A & B. Transverse section of healthy tobacco stem. B/AB/180/60μm.

A. Light micrograph.

B. Fluorescence micrograph. Note the strong autofluorescence in the xylem (X) and sclerenchyma (S), and weaker fluorescence in the external (eP) and internal (iP) phloem.

C & D. Transverse section of tobacco stem 60 days after inoculation of roots with *Olpidium*. B/AB/150/60μm.

C. Light micrograph.

D. Fluorescence micrograph. Strong autofluorescence in the xylem (X), and fluorescence in the phloem (eP & iP).

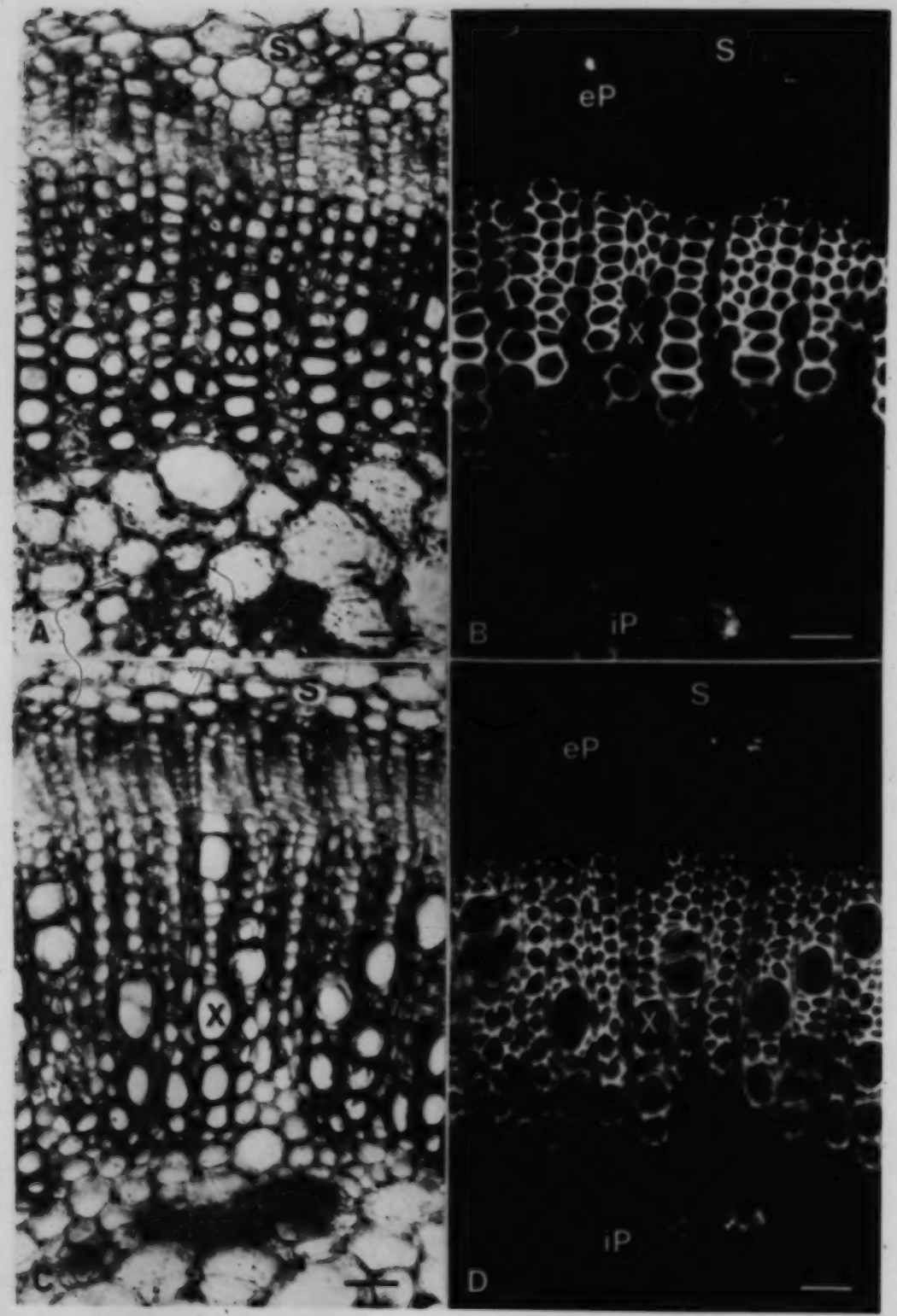

S
A
S
eP
X
B
iP
S
C
X
S
eP
X
iP
D

PLATE 23. Light and fluorescence micrographs of tobacco stems.

A & B. Transverse section of tobacco stem showing stunt symptoms 60 days after inoculation of the roots with *Olpidium*/TSA. B/AB/200/60µm.

A. Light micrograph. Cell walls of xylem are irregularly thickened. Dark material present in xylem tissues (arrows). Internal phloem (iP) is more extensive than in healthy and *Olpidium* controls.

B. Fluorescence micrograph. Autofluorescence in xylem is irregular. Fluorescence in phloem tissues is apparently normal.

C & D. Transverse section of tobacco stem showing stunt 30 days after sap inoculation with TSA. B/AB/170/60µm.

C. Light micrograph. Extensive internal (iP) and external (eP) phloem present. Xylem (X) showing some irregularity.

D. Fluorescence micrograph. Fluorescence in phloem tissues is normal in distribution but increased in intensity when compared with controls. Autofluorescence in the xylem shows some irregularities.

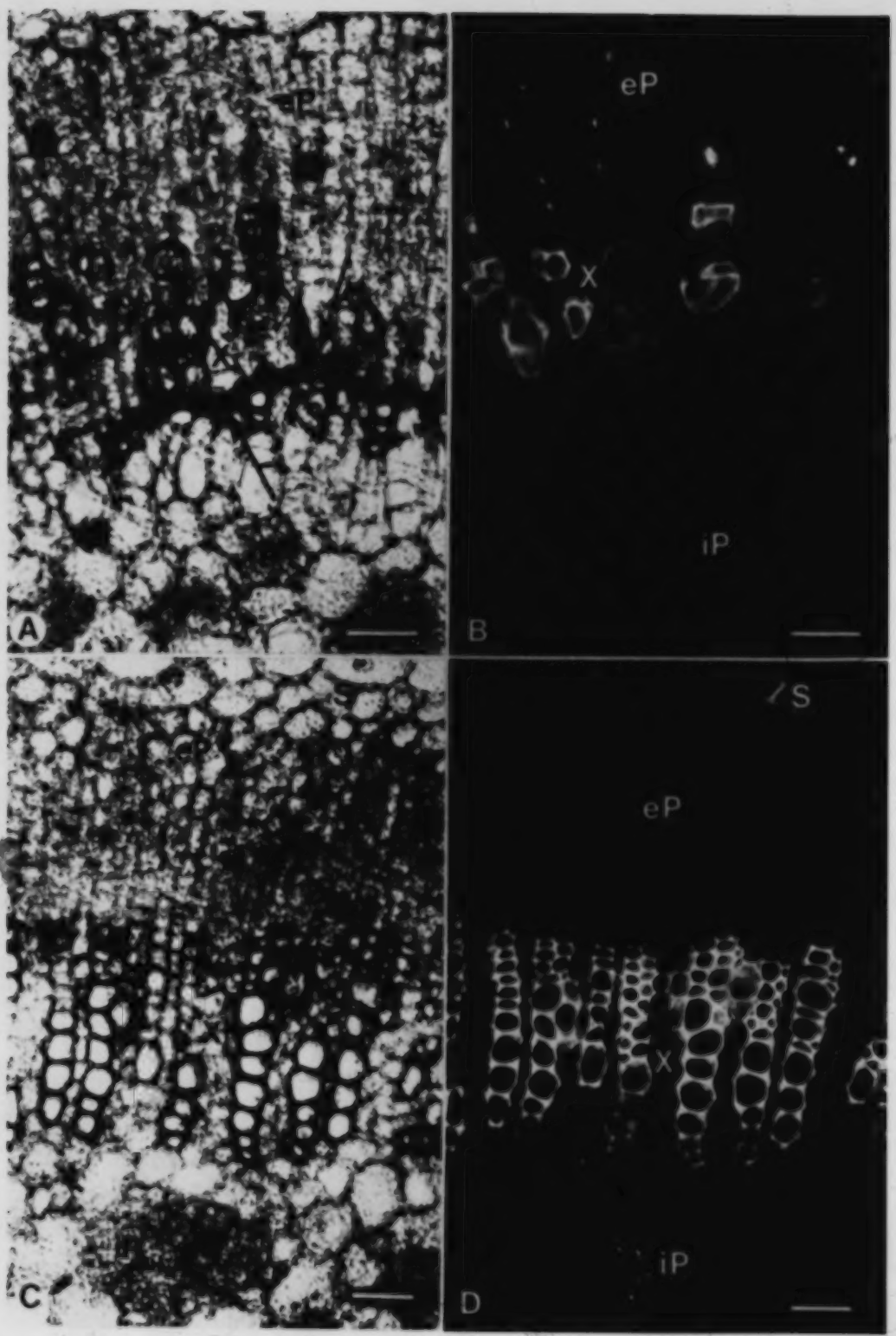

eP
X
iP
A
B
S
eP
X
iP
C
D

PLATE 24. Light and fluorescence micrographs of tobacco stems.

A - D. Longitudinal sections. B/AB/160/100μm.

A. Light micrograph of healthy tobacco.

B. Fluorescence micrograph of same tissues as in A. Note the strong autofluorescence associated with the xylem (X), and the weaker fluorescence in the phloem (eP & iP).

C. Light micrograph of tobacco stem showing stunt symptoms 60 days after inoculation of the roots with *Olpidium*/TSA. Note the disorganisation of the xylem (X) and phloem (iP) as compared to the healthy stem in A.

D. Fluorescence micrograph of same tissues as in C. Note the weak fluorescence in the xylem (X), and the increase in the intensity of fluorescence in the phloem (iP) but with apparently normal distribution.

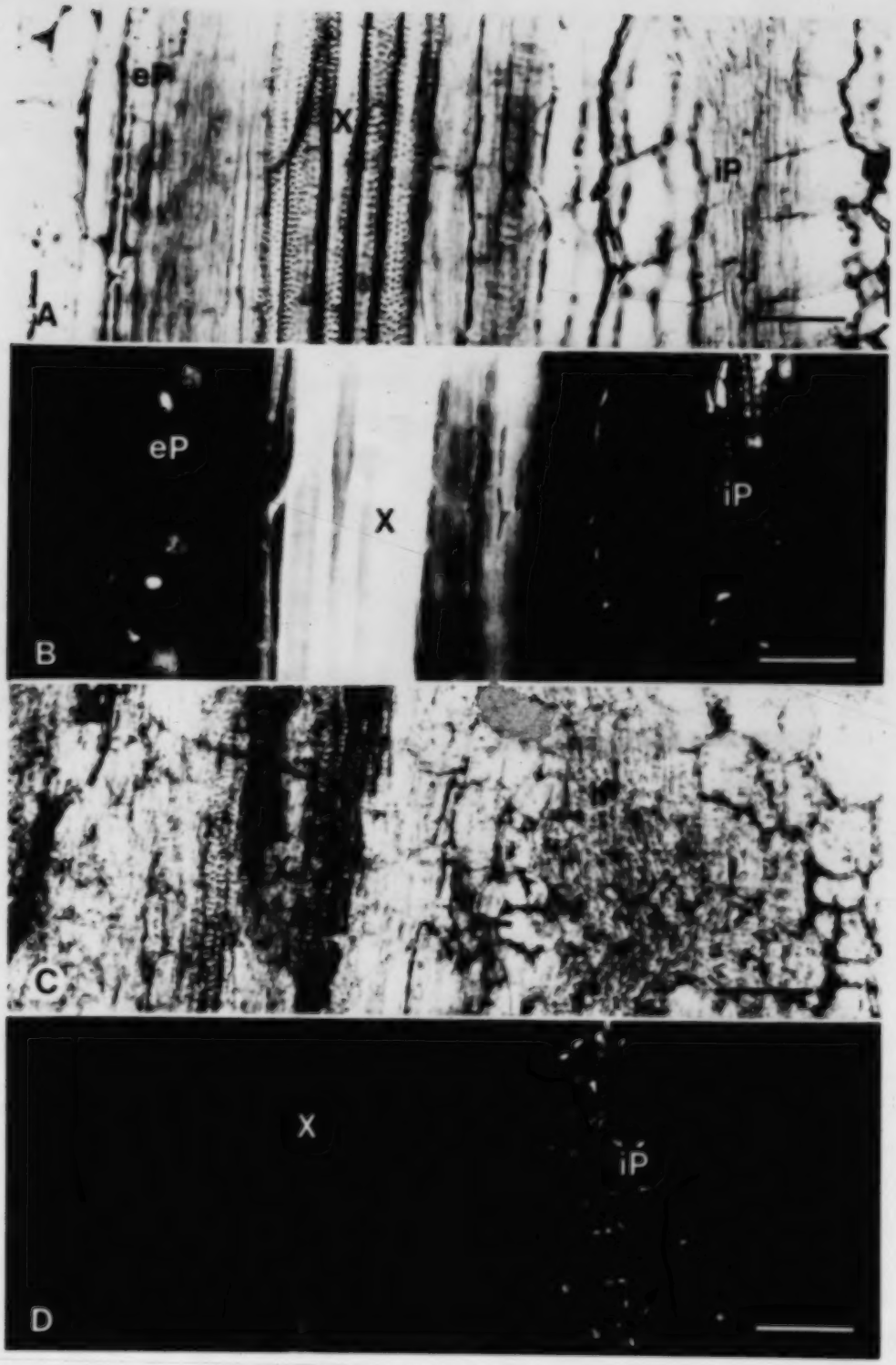

eP
X
iP
A
eP
X
iP
B
C
X
iP
D

PLATE 25. Scanning electron micrographs of transverse sections of tobacco stems.

A & B. Healthy tobacco, 8 weeks old.

A. Whole stem. F/*/18/750µm.

B. Enlargement of vascular tissues in A. F/*/90/150µm.

C & D. Tobacco, 8 weeks old, 6 weeks after roots inoculated with *Olpidium*.

C. Whole stem. F/*/22/750µm.

D. Enlargement of vascular tissues in C. F/*/90/150µm.

E & F. Tobacco, 8 weeks old, 6 weeks after roots inoculated with *Olpidium*/TSA.

E. Whole stem. F/*/18/750µm.

F. Enlargement of vascular tissues in E. Note the disorganisation of the xylem, and the presence of extensive internal (iP) and external (eP) phloem. F/*/90/150µm.

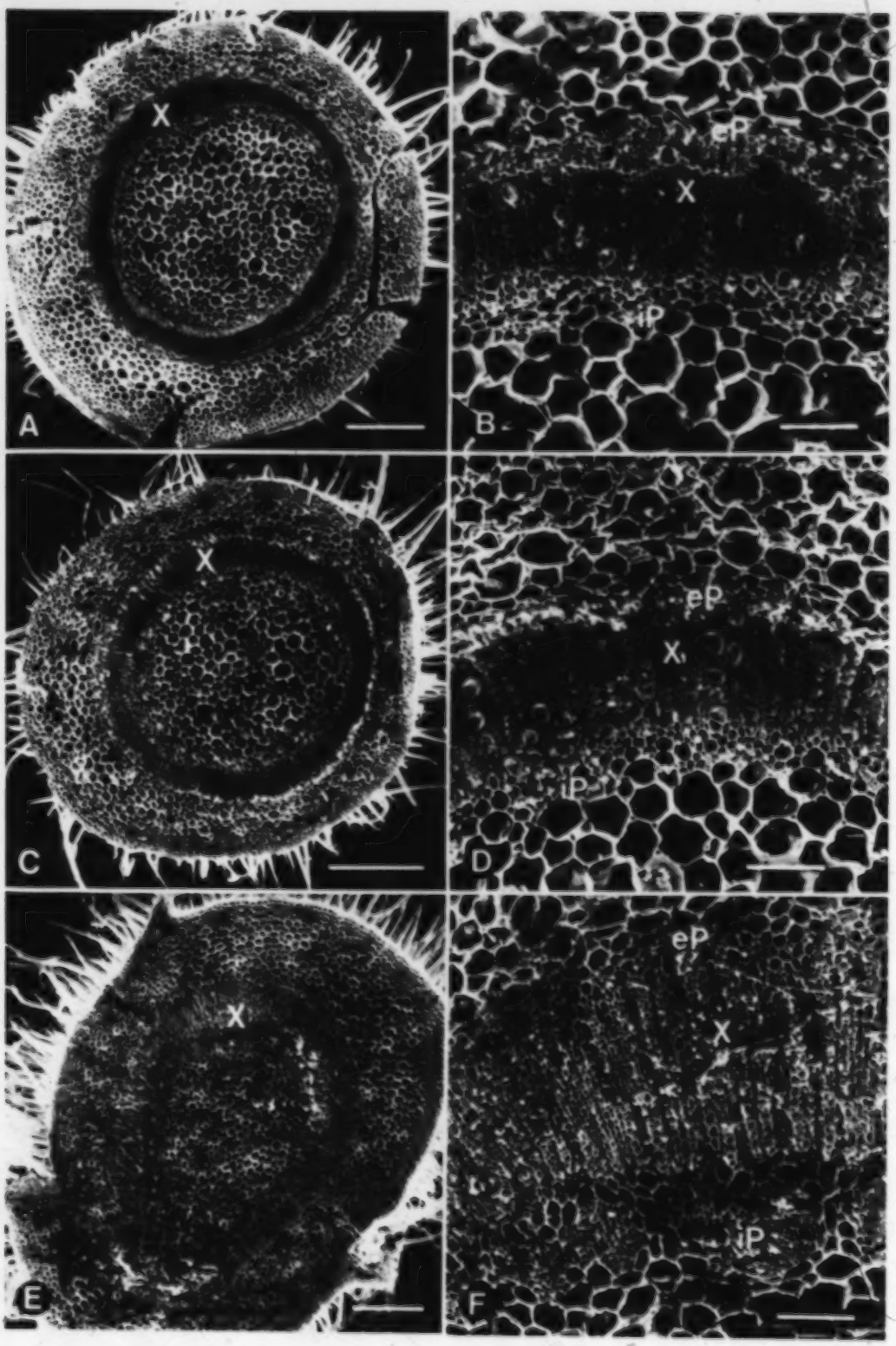

X
A
ep
X
ip
B
X
C
ep
X
ip
D
X
E
ep
X
ip
F

PLATE 26. Scanning electron micrographs of transverse sections of tobacco stems.

A & B. Xylem cells. F/*/450/30μm.

A. Healthy tobacco.

B. Tobacco 6 weeks after inoculation of roots with *Olpidium*/TSA.

C & D. Xylem cells. F/*/1,800/10μm.

C. Tobacco 6 weeks after inoculation of roots with *Olpidium*. Note the lignified cell walls.

D. Tobacco 6 weeks after inoculation of roots with *Olpidium*/TSA. Note the thin cell walls.

E & F. Internal phloem cells. F/*/450/30μm.

E. Healthy tobacco.

F. Tobacco 6 weeks after inoculation of roots with *Olpidium*/TSA. Note the extensive development of phloem, but no apparent necrosis.

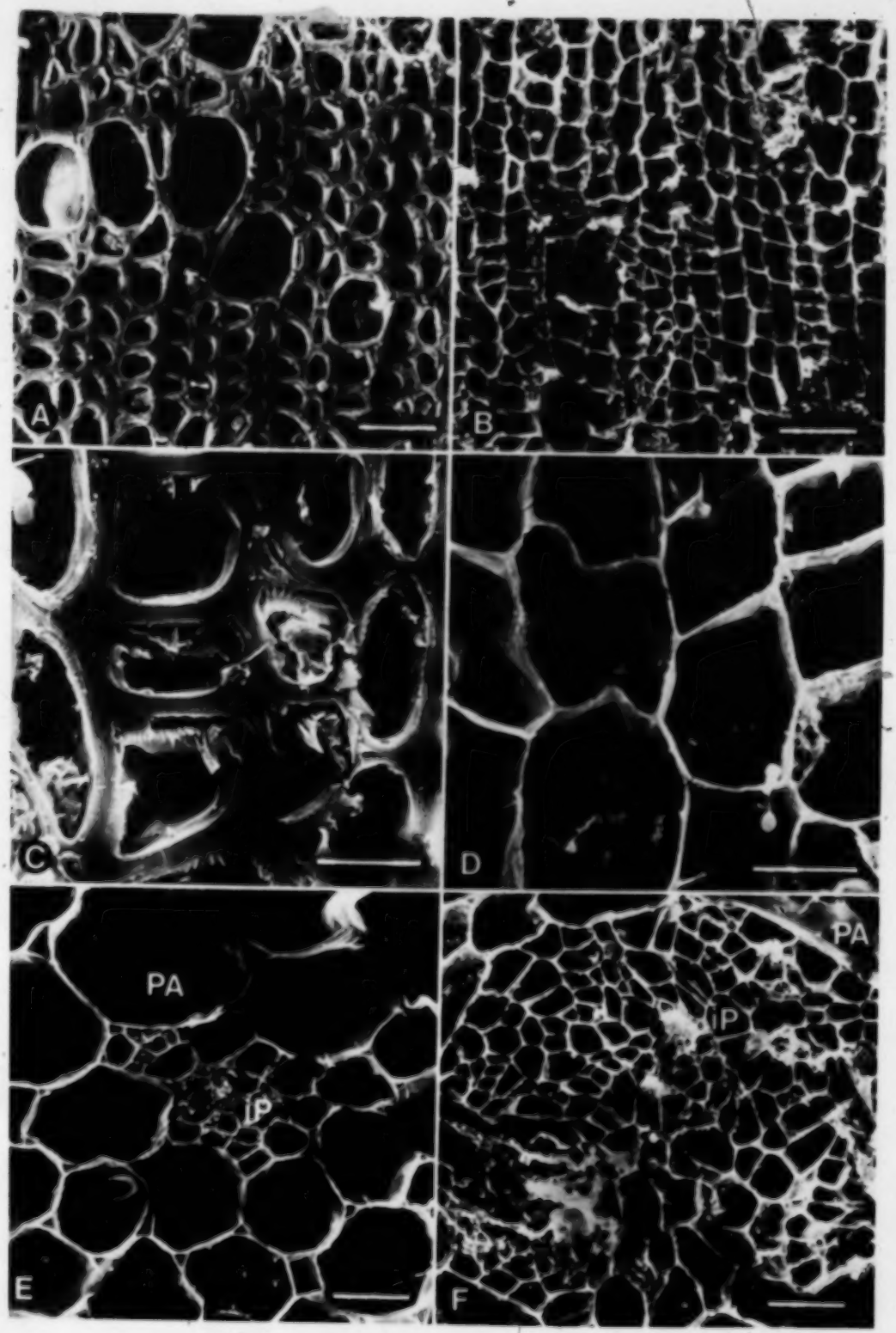

A
B
C
D
PA
IP
E
PA
IP
F

PLATE 27. Scanning electron micrographs of transverse sections of tobacco tissues.

A, B & C. Petiole of tobacco 6 weeks after inoculation of roots with *Olpidium*/TSA. Note the dense gum-like material.

A. Vascular tissues of petiole. F/*/90/150μm.

B. Enlargement of region (large arrow) in A to show the relationship of the gum-like material to the cells. F/*/1,800/10μm.

C. Same petiole section as in A viewed obliquely to show the gum-like material (arrows) on the cut surface. F/*/450/30μm.

D, E & F. Xylem of tobacco stem 6 weeks after inoculation of roots with *Olpidium*/TSA.

D. Disorganised xylem with thin-walled cells and gum-like material (arrows). F/*/450/30μm.

E. Thin-walled and lignified xylem cells together. Necrotic cells contain gum-like material (arrows). FG+O,F/*/450/10μm.

F. Enlargement of gum-like material in xylem cell in E. FG+O,F/*/1,800/10μm.

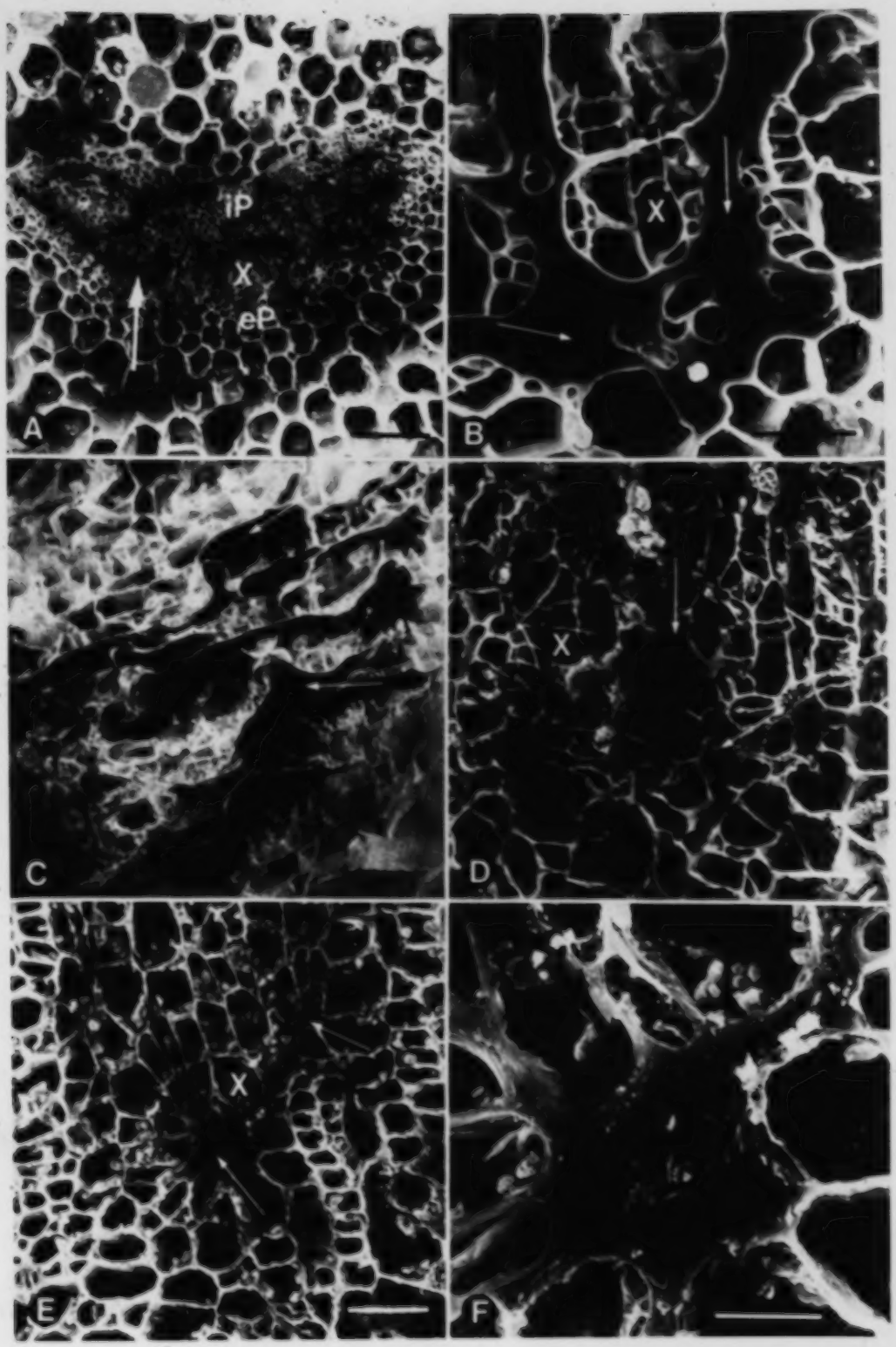

iP
X
eP
A
X
B
C
X
D
X
E
F

PLATE 28. Transmission electron micrographs of vascular tissues in tobacco stems.

A & B. Transverse sections of phloem in tobacco showing stunt symptoms 60 days after inoculation of the roots with *Olpidium*/TSA.

A. Sieve cells (SC) and companion cells (C) in phloem tissues. FG+O/UA+LC/3,700/4μm.

B. Sieve cells (SC) separated by a sieve plate (SP). The cells are apparently normal, and contain P-protein (PP) and plastids (PL). FG+O/UA+LC/18,400/1μm.

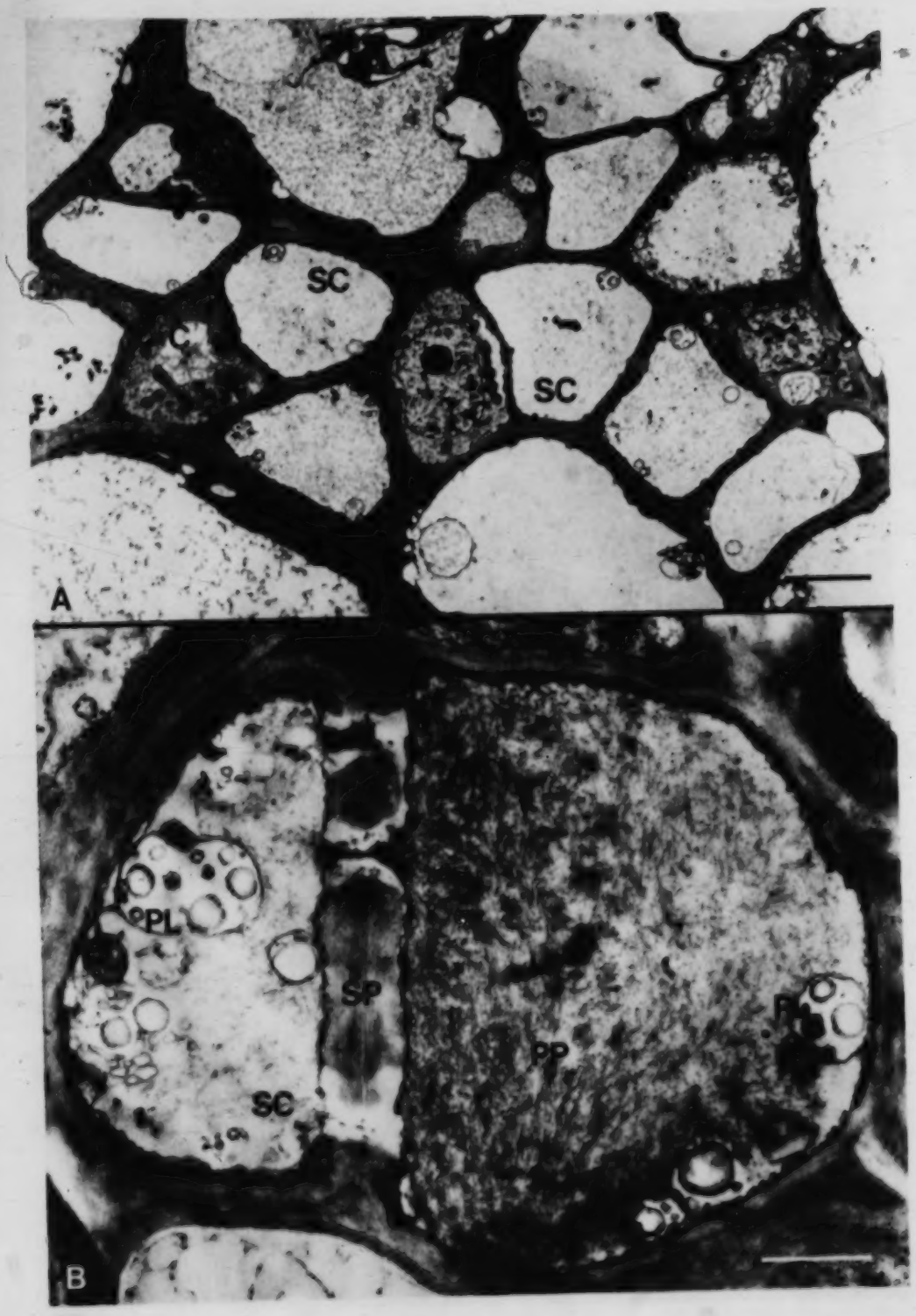

SC
SC
A
PL
SP
PP
SC
B

PLATE 29. Transmission electron micrographs of tissues infected with tobacco stunt agent.

A & B. Transverse sections of xylem in tobacco stems. FG+O/UA+LC/3,700/3μm.

A. Xylem cells in healthy tobacco. Xylem vessel (XV) with lignified wall, and cells containing cytoplasm.

B. Xylem cells in tobacco showing stunt symptoms 60 days after inoculation of the roots with *Olpidium*/TSA. Cell walls are thin and have irregular outlines. Cytoplasm inside most of the cells has degenerated. Parts of the lignified walls of two xylem vessels (XV) are visible.

C. Chloroplasts (CH) in a mesophyl cell of a tobacco leaf showing stunt symptoms. Note the large granules of starch (ST). The lower chloroplast has a disorganised membrane system due to the starch accumulation. FG+O/UA+LC/17,600/1μm.

D. Chloroplasts (CH) in a mesophyl cell of a leaf of *Chenopodium amaranticolor* with 3 days old necrotic lesions after inoculation with tobacco sap containing stunt agent. Note the large starch (ST) granules within the chloroplasts. The chloroplast at the lower right has degenerated. FG+O/UA+LC/15,000/1μm.

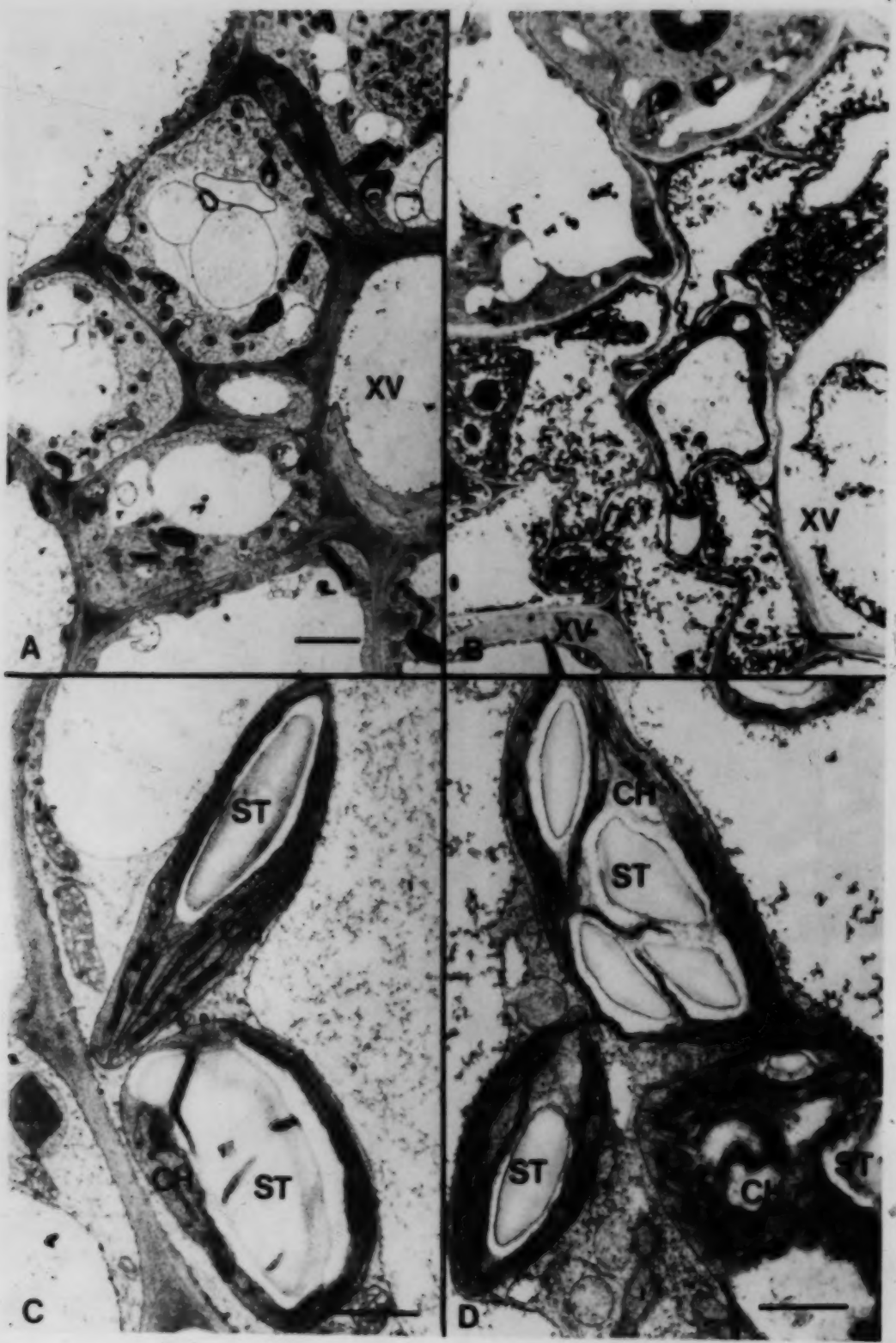
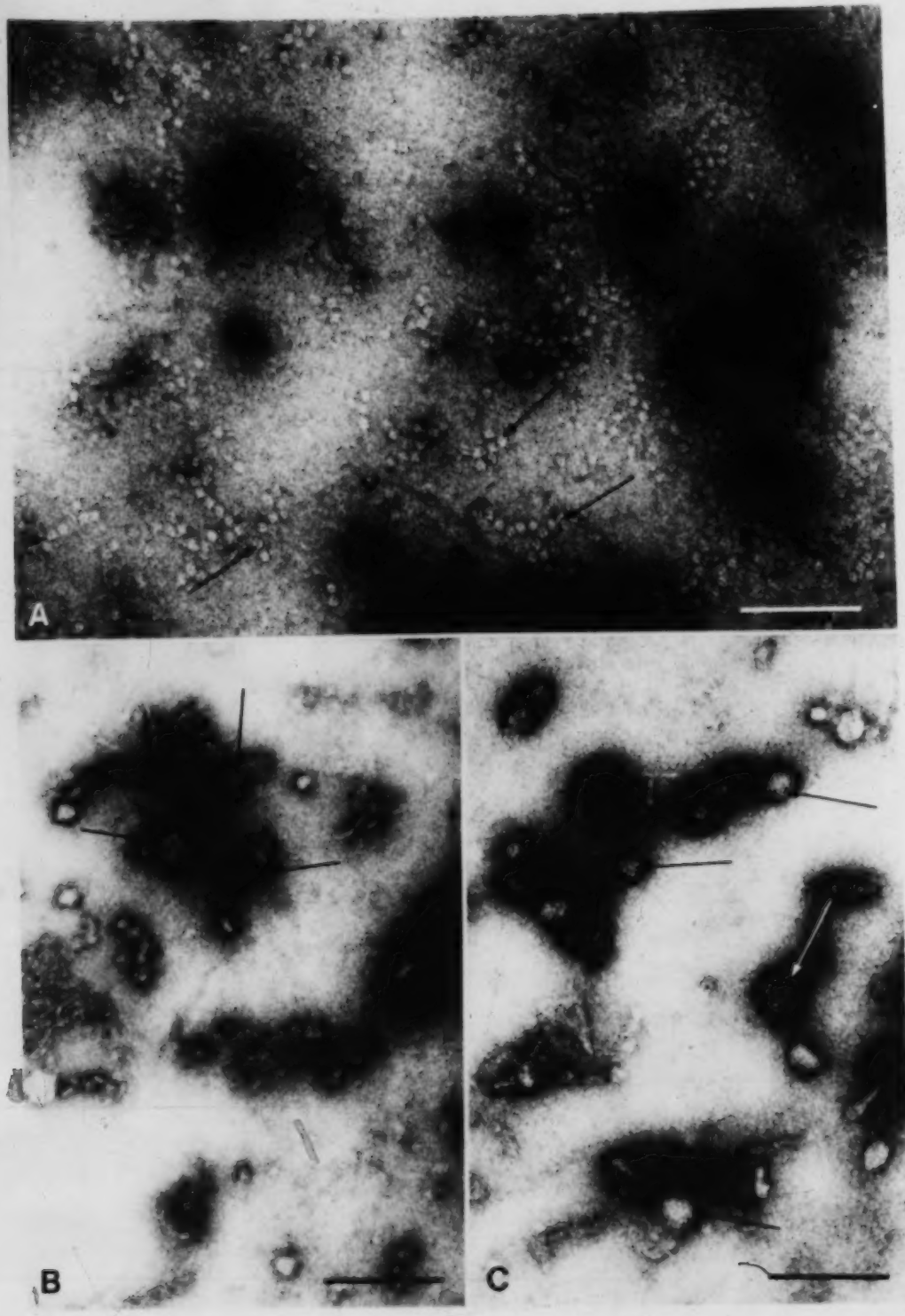

XV
A
XV
XV
B
ST
CH
ST
C
CH
ST
ST
CH
ST
D

PLATE 30. Transmission electron micrographs of negative stained tobacco stunt agent preparations.

A - C. */PTA/200,000/0.1μm.

A. A preparation obtained after two cycles of differential centrifugation. Particles (arrows) approximately 9 nm in diameter. No infectivity associated with sample.

B & C. Preparations obtained after two cycles of polyethylene glycol 6000 precipitation. No infectivity associated with samples. Particles (arrows) range in size from 15 to 35 nm. Some particles (white arrow) observed with a possible membrane.


A
B
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APPENDIX

Analysis of lesion counts obtained from control and treatment inoculations made on the 2 halves of the same leaves of *Chenopodium amaranticolor*.

t-test of significance for correlated samples (Snedecor & Cochran, 1967)

$$t = \frac{\Sigma D}{\sqrt{\left(\frac{N\Sigma D^2 - (\Sigma D)^2}{N - 1}\right)}}$$

where D = difference between paired samples.

N = number of paired samples.

Significance at the 1% level (α .01) denoted by **

Significance at the 5% level (α .05) denoted by *

No significance denoted by NS

TABLE 5. Lesion counts: Effect of buffer pH on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	Buffer pH					
	5.0	5.5	6.5	7.0	7.5	8.5
	271	261	210	321	374	698
	274	452	590	732	620	376
Exp. I	366	537	755	291	363	470
	443	291	652	644	517	223
Lesion	185	609	419	303	309	239
counts	362	460	602	758	272	270
	393	512	134	697	984	548
	161	431	883	482	766	490
Total	2455	3553	4245	4228	4205	3314
Mean	307	444	531	529	526	414
	43	183	150	262	178	136
	10	175	117	269	151	100
Exp. II	70	41	276	118	320	160
	47	134	354	197	92	153
Lesion	33	257	98	190	285	198
counts	74	174	210	402	128	149
	63	217	236	244	148	287
	12	159	169	118	164	229
Total	352	1340	1610	1800	1466	1412
Mean	44	168	201	225	183	177

TABLE 6. Lesion counts: Effect of buffer molarity on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor* Exp. I

	Buffer molarity			
	0.5	0.05	0.01	0.005
	51	267	357	304
	53	231	213	188
	12	317	175	175
	45	292	231	170
	34	193	164	210
	41	271	359	243
	5	249	321	267
Lesion	0	212	364	249
counts	11	172	159	63
	33	174	190	95
	9	219	268	281
	13	235	224	229
	4	120	193	217
	26	298	251	232
	7	241	345	180
	12	307	278	193
Total	356	3798	4092	3296
Mean	22	237	256	206

TABLE 7. Lesion counts: Effect of buffer molarity on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor* Exp. II

	Buffer molarity							
	0.5	0.1	0.05	0.01	0.005	0.001	0.01M (no 4-PTC)	distilled H_2O
	14	133	247	482	217	435	64	116
	12	56	236	524	480	338	24	88
	38	193	267	525	296	408	53	30
	17	244	100	577	586	556	58	14
	34	175	325	435	349	327	50	108
	42	191	290	483	289	372	69	22
	15	72	299	165	536	494	140	63
	25	36	273	522	814	417	172	63
Total	197	1100	2037	3713	3567	3347	630	504
Mean	25	138	255	464	446	418	79	63

TABLE 8. Lesion counts: *Chenopodium amaranticolor* inoculated with tobacco stunt agent in tobacco sap and incubated at 17°C

Leaf No.	Days after inoculation										
	4	5	6	7	8	9	10	12	14	16	18
1	0	0	1	26	62	80	91	106	119	128	128
2	0	0	0	4	34	59	72	101	118	135	138
3	0	0	1	10	24	36	50	65	86	91	102
4	0	0	0	5	14	27	31	45	67	83	86
5	0	0	0	46	96	118	139	153	169	169	169
6	0	0	0	50	121	194	231	270	318	341	341
7	0	0	2	26	85	117	144	211	245	253	253
8	0	0	9	40	90	131	161	198	218	241	241
9	0	0	0	5	21	62	73	105	110	111	111
10	0	0	0	9	42	72	89	103	121	123	123
11	0	0	0	24	58	90	131	158	182	198	198
12	0	0	1	5	24	41	54	76	90	108	108
13	0	0	0	0	5	14	17	29	42	45	46
14	0	0	0	1	10	32	38	68	86	96	102
15	0	0	0	14	42	65	78	104	134	142	142
16	0	0	0	15	42	82	109	146	197	221	221
Total	0	0	14	280	770	1220	1508	1938	2302	2486	2510
Mean	0	0	1	18	48	76	94	121	144	155	157

TABLE 9. Lesion counts: *Chenopodium amaranticolor* inoculated with tobacco stunt agent in tobacco sap and incubated at 21°C

	Days after inoculation									
Leaf No.	4	5	6	7	8	9	10	12	14	16
1	0	51	75	99	113	131	137	142	145	145
2	0	43	74	111	126	151	165	185	185	185
3	0	22	33	46	63	70	82	90	94	98
4	0	25	63	119	157	185	193	213	222	222
5	0	11	40	80	103	114	120	124	124	124
6	0	4	14	333	42	47	53	62	62	62
7	0	26	107	181	214	227	240	252	252	252
8	0	6	24	43	61	64	67	82	82	82
9	0	75	121	139	165	169	169	171	171	171
10	0	47	64	86	110	124	126	130	130	130
11	0	20	32	44	67	80	85	101	101	102
12	0	21	44	66	98	114	127	146	159	159
13	0	5	13	24	35	39	40	42	45	45
14	0	71	107	169	195	209	224	242	255	255
15	0	108	167	235	269	291	294	310	310	310
16	0	46	74	105	124	134	142	149	149	150
Total	0	581	1052	1580	1947	2149	2264	2441	2484	2490
Mean	0	36	66	99	122	134	142	153	155	156

TABLE 10. Lesion counts: *Chenopodium amaranticolor* inoculated with tobacco stunt agent in tobacco sap and incubated at 25°C

	Days after inoculation									
Leaf No.	4	5	6	7	8	9	10	12	14	16
1	0	48	69	104	126	134	135	136	136	136
2	0	17	31	53	71	78	83	85	85	85
3	0	16	47	71	97	108	121	126	128	128
4	0	27	84	138	180	191	191	192	192	192
5	0	3	11	20	32	38	47	53	60	60
6	0	0	1	9	12	18	24	28	28	28
7	0	0	6	18	28	38	51	68	86	87
8	0	2	18	30	46	74	83	107	114	114
9	0	10	22	41	48	55	59	61	62	62
10	0	15	27	42	59	72	81	89	93	95
11	0	2	13	33	51	60	71	83	89	89
12	0	4	16	29	39	54	73	87	94	94
13	0	1	15	34	49	54	59	66	66	66
14	0	2	9	19	28	37	45	50	54	56
15	0	1	13	33	47	50	67	78	85	85
16	0	0	3	8	13	19	24	30	31	31
Total	0	148	385	679	923	1074	1208	1335	1403	1408
Mean	0	9	24	42	58	67	76	83	88	88

TABLE 11. Lesion counts: Increment curves I & II of tobacco stunt agent infectivity in tobacco bioassayed on *Chenopodium amaranticolor* after *Olpidium* transmission

	Days after inoculation													
	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Exp.I	0	0	1	8	16	84	126	43	60	43	107	119	5	2
	0	0	0	8	32	88	108	68	18	55	64	54	6	16
Lesion	0	0	0	10	34	40	248	232	43	32	23	30	2	10
counts/	0	0	1	17	35	267	120	103	98	105	25	34	4	8
leaf	0	0	1	4	27	112	96	128	53	80	72	49	3	2
	0	0	0	10	50	128	110	109	43	73	70	19	4	3
	0	0	0	5	20	68	227	244	162	64	67	62	2	1
	0	0	0	12	25	84	276	268	142	65	102	67	3	2
Total	0	0	3	74	239	871	1391	1255	619	517	530	434	29	44
Mean	0	0	0	9	30	109	174	157	77	65	66	54	4	6
Exp.II	0	0	0	42	84	241	324	307	153	207	133	103	75	72
	0	0	7	49	135	185	105	165	229	93	154	179	101	34
Lesion	0	0	15	38	98	169	82	179	89	98	107	68	183	47
counts/	0	0	0	33	45	191	297	204	196	115	91	134	124	57
leaf	0	0	2	45	99	199	163	176	173	176	124	109	84	48
	0	0	19	12	103	77	290	337	212	183	127	77	83	23
	0	0	9	26	165	75	347	131	142	151	102	81	76	45
	0	0	12	32	62	96	439	167	188	195	89	98	91	74
Total	0	0	64	277	791	1233	2047	1666	1382	1218	927	849	817	400
Mean	0	0	8	33	99	154	256	208	173	152	116	106	102	50

TABLE 12. Lesion counts: Increment curve I of tobacco stunt agent infectivity in tobacco bioassayed on *Chenopodium amaranticolor* after sap transmission

	Days after inoculation										
	4	6	8	10	12	14	16	18	20	22	24
Inoc.	4	74	184	122	262	93	99	237	79	102	88
leaves	1	86	116	186	103	273	82	134	81	66	48
	1	79	215	267	204	128	202	138	106	135	57
Lesion	1	145	201	175	262	152	119	112	185	102	67
counts/	0	59	136	157	233	108	196	80	126	56	37
leaf	2	77	143	181	207	109	108	127	149	164	101
	0	28	141	133	376	211	175	204	138	68	45
	0	21	93	144	116	315	173	119	127	112	63
Total	9	569	1229	1365	1763	1389	1154	1151	991	805	506
Mean	1	71	154	170	220	174	144	144	124	101	63
Non-	0	0	0	71	87	142	93	139	52	69	47
inoc.	0	0	0	69	65	208	159	207	113	11	12
leaves	0	0	0	99	85	142	317	180	99	72	27
	0	0	0	109	196	175	378	95	78	96	29
Lesion	0	0	0	70	76	25	228	78	77	56	49
counts/	0	0	0	46	86	96	227	53	57	70	23
leaf	0	0	0	18	58	113	440	185	65	78	53
	0	0	0	124	217	223	184	204	129	81	67
Total	0	0	0	606	870	1124	2026	1141	670	533	307
Mean	0	0	0	76	109	141	253	143	84	67	38

TABLE 13. Lesion counts: Increment curve II of tobacco stunt agent infectivity in tobacco bioassayed on *Chenopodium amaranticolor* after sap transmission

	Days after inoculation									
	4	6	8	10	12	14	16	18	20	22
Inoc.	0	4	54	45	16	139	59	27	71	31
leaves	0	4	86	69	78	92	7	30	45	38
	0	2	12	65	75	156	7	195	37	33
Lesion	1	0	104	31	102	101	31	121	23	3
counts/	1	12	38	18	12	48	155	58	44	47
leaf	1	4	43	85	209	65	166	62	52	28
	0	3	36	62	80	31	102	51	65	93
	0	3	27	86	52	160	117	23	97	42
Total	3	32	400	461	624	792	644	567	434	315
Mean	0	4	50	58	78	99	81	71	54	39
Non-	0	0	0	0	13	36	171	308	89	117
inoc.	0	0	0	0	17	69	96	158	116	100
leaves	0	0	0	0	3	43	279	231	78	168
	0	0	0	0	7	72	84	116	66	45
Lesion	0	0	0	0	11	91	88	52	206	207
counts/	0	0	0	0	5	219	169	274	273	82
leaf	0	0	0	0	7	59	63	111	341	169
	0	0	0	0	10	151	159	197	156	193
Total	0	0	0	0	73	740	1109	1447	1325	1081
Mean	0	0	0	0	9	93	139	181	166	135

TABLE 14. Lesion counts: Effects of anti-oxidants and antibiotics on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

Control	1% ascorbic acid	Control	1% sodium sulphite	Control	1,000 ppm Tetracycline	Control	10,000 I.U. Penicillin	Control	1,000 ppm Chloram-phenicol
130	28	91	35	118	61	45	43	194	101
97	10	135	16	194	78	90	44	92	38
154	47	144	39	40	29	30	57	43	48
143	24	47	13	87	86	96	61	68	48
151	54	139	16	102	89	93	67	49	68
101	20	45	6	82	43	169	161	30	29
63	16	105	24	144	26	146	174	62	55
89	12	164	55	160	81	112	102	73	17
112	2	108	97	19	23	69	46	33	7
61	8	42	49	7	43	55	40	135	196
98	5	93	95	8	19	21	55	86	42
58	1	54	48	22	29	52	44	44	95
112	11	52	42	47	20	118	102	50	95
62	17	53	114	27	7	101	32	14	92
85	6	60	91	29	9	138	168	30	112
129	15	89	120	14	5	81	32	89	102
Total 1645	276	1421	860	1100	648	1416	1228	1092	1145
Mean 103	17	89	54	69	41	89	77	68	72
t = 14.1 **(α .01)		t = 2.40 *(α .05)		t = 2.56 *(α .05)		t = 1.55 NS		t = -0.25 NS	

TABLE 15: Lesion counts: Effects of RNase, DNase and protease on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	Control	5 µg/ml RNase	Control	100 µg/ml DNase	Control	500 µg/ml Protease	Control	1,000 µg/ml Protease	Control	2,000µg/ml Protease
	99	0	135	110	106	33	136	6	161	5
	124	0	174	69	140	35	95	8	143	6
	142	0	259	180	49	10	84	8	230	1
	89	0	193	212	71	15	152	4	245	2
	56	0	46	96	167	68	82	3	119	1
	64	0	114	152	273	73	143	7	169	1
	154	0	231	179	224	60	153	4	145	0
	96	0	138	192	250	59	169	8	114	0
	48	0	110	85	130	101	152	5	253	6
	94	0	92	179	108	30	228	14	99	3
	89	0	168	97	203	57	202	9	69	11
	60	0	164	121	214	45	82	6	260	1
	124	0	15	42	96	39	50	12	141	1
	51	0	40	47	92	59	100	5	175	0
	185	0	24	36	139	71	154	5	38	1
	50	0	92	47	109	54	185	3	91	0
Total	1525	0	1995	1844	2371	809	2167	107	2452	39
Mean	95	0	125	115	148	51	135	7	153	2
	t = 9.25 **(α.01)		t = 0.69 NS		t = 6.73 **(α.01)		t = 3.43 **(α.01)		t = 8.99 **(α.01)	

TABLE 16. Lesion counts: Effect of RNase concentration on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	RNase concentration					
	1 µg/ml	0.1 µg/ml	0.01 µg/ml	0.001 µg/ml	0.0001 µg/ml	* Control
	0	2	79	163	123	166
	0	1	24	105	190	25
	0	0	97	155	103	115
Lesion	0	0	68	54	125	191
counts/	0	0	9	43	102	62
leaf	0	1	91	78	14	140
	0	0	92	122	50	111
	0	0	9	58	111	29
	0	0	73	51	201	78
	0	0	69	98	76	115
	0	1	42	107	127	132
	0	1	58	92	118	183
	0	0	47	133	94	98
	0	0	53	129	87	67
	0	0	81	93	71	64
	0	1	36	79	48	91
Total	0	7	928	1560	1640	1667
Mean	0	0	58	98	103	104

*Control = 0.02 M phosphate + 0.001 M 4-PTC pH 7.0 + equal volume of distilled water

TABLE 17. Lesion counts: Kinetics of RNase treatment on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

		Incubation time (minutes)											
		0		10		20		30		40		60	
	*	C	T	C	T	C	T	C	T	C	T	C	T
		68	64	37	6	15	8	11	4	18	5	12	2
0.001 µg/ml		152	41	33	5	22	2	25	8	3	3	7	0
RNase		85	44	31	6	4	11	20	8	9	3	2	0
	Lesion	49	15	14	11	3	1	7	0	6	1	2	0
	counts/	58	18	15	2	29	11	11	4	14	1	1	0
	half leaf	10	8	32	5	6	11	12	0	7	1	12	1
		76	11	12	2	31	1	25	1	8	2	11	1
		41	16	21	1	20	1	5	2	7	4	5	1
	Total	539	217	195	38	130	46	116	27	72	20	52	5
	Mean	67	27	24	5	16	6	15	3	9	3	7	1

(Continued ....)

TABLE 17. Lesion counts: Kinetics of RNase treatment on TSA infectivity in tobacco sap (Continued)

		Incubation time (minutes)											
		0		10		20		30		40		60	
	*	C	T	C	T	C	T	C	T	C	T	C	T
		115	88	110	15	69	22	37	4	31	6	19	5
0.01 µg/ml		133	92	59	31	77	24	38	5	22	6	8	0
RNase		165	131	198	13	83	6	88	13	28	6	4	1
	Lesion	253	51	29	20	70	13	70	1	40	3	7	2
	counts/	85	42	47	42	87	12	23	8	7	3	8	0
	half leaf	80	30	85	14	68	6	49	5	33	0	16	0
		139	46	119	22	114	11	15	3	45	0	6	1
		129	37	142	21	115	13	30	4	21	0	6	0
	Total	1099	517	789	178	683	107	350	43	227	24	74	9
	Mean	137	65	99	22	85	13	44	5	28	3	9	1

*C = control i.e. 0.01 M phosphate pH 7.0 + 0.001 M 4-PTC

T = treatment i.e. phosphate/PTC buffer + RNase

TABLE 18. Lesion counts: Effect of bentonite on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	Mg - bentonite					
	Control	2.5 mg/ml	Control	5 mg/ml	Control	12 mg/ml
	89	84	33	7	62	0
	104	90	14	1	42	0
Lesion	47	93	19	12	25	0
counts/	130	134	12	3	4	0
half leaf	126	84	9	4	10	0
	78	95	17	46	7	0
	81	86	5	21	27	0
	19	46	17	8	88	0
Total	674	712	126	102	265	0
Mean	84	89	16	13	33	0
	t =-0.50 NS		t = 0.48 NS		t = 3.17 *(α.05)	

	Bentonite					
	Control	3 mg/ml	Control	8 mg/ml	Control	16 mg/ml
	98	20	32	1	17	0
	63	34	26	0	26	0
Lesion	122	9	7	1	8	0
counts/	159	44	50	0	7	0
half leaf	40	37	121	2	21	0
	65	49	262	0	21	0
	155	72	29	2	4	0
	255	121	50	2	83	0
Total	957	386	577	8	187	0
Mean	120	48	72	1	23	0
	t = 4.06 **(α.01)		t = 2.39 *(α.05)		t = 2.60 *(α.05)	

TABLE 19. Lesion counts: Effect of the addition of yeast RNA on tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	Yeast RNA concentration (mg/ml)				
	0.1	1	5	10	100
	4	1	18	4	0
Lesion	3	1	5	8	0
counts/	1	7	23	19	1
leaf	2	9	24	12	0
	3	9	6	29	0
	3	5	22	34	0
	1	6	19	6	1
	1	8	31	6	1
	2	1	51	16	0
	8	9	5	30	0
	0	5	4	17	0
	0	8	18	31	0
Total	28	72	-222	212	3
Mean	2	6	19	18	0

TABLE 20. Lesion counts: Effect of yeast-RNA and Mg^{2+} on tobacco stunt agent infectivity in tobacco sap incubated at 20°C and bioassayed on *Chenopodium amaranticolor*

		Incubation time (minutes)									
		0		10		20		30		60	
	*	C	T	C	T	C	T	C	T	C	T
Buffer		78	212	11	138	32	140	34	168	1	77
+		75	61	50	103	10	36	13	134	2	79
5 mg yeast		261	308	83	228	11	101	37	93	1	70
RNA/ml		126	102	139	125	45	31	79	237	7	108
	Lesion	15	39	13	44	23	74	1	99	8	33
	counts/	8	70	14	90	38	182	4	38	2	19
	half leaf	132	415	25	156	65	200	25	112	1	25
		137	244	10	116	32	230	36	122	9	79
	Total	832	1451	345	1000	256	994	229	1003	31	490
	Mean	104	181	43	125	32	124	29	125	4	61
Buffer		139	89	85	77	16	3	9	15	1	4
+		80	99	29	48	18	3	22	10	7	3
0.01 M		147	264	44	70	100	79	27	32	6	4
$MgCl_2$		267	186	84	260	30	66	20	33	5	2
	Lesion	52	12	46	79	5	13	23	10	1	1
	counts/	58	50	14	23	21	37	4	3	0	1
	half leaf	28	30	36	12	49	84	13	16	6	4
		60	153	98	28	36	9	3	4	14	4
	Total	831	883	436	597	275	294	121	123	40	23
	Mean	104	110	55	75	34	37	15	15	5	3

*C = control i.e. 0.01 M phosphate pH 7.0 + 0.001 M 4-PTC

T = treatment i.e. phosphate/PTC + yeast RNA or $MgCl_2$

TABLE 21. Lesion counts: Dilution end point of tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

		Dilution (sap: buffer)				
		1:1	1:5	1:10	1:50	1:100
Exp. I		152	180	117	1	0
	Lesion	461	219	61	1	0
	counts/	474	113	56	2	0
	leaf	298	204	170	0	0
		293	244	60	0	0
		436	310	88	1	0
		422	89	75	0	0
		277	105	75	1	0
	Total	2813	1464	702	6	0
	Mean	352	183	88	1	0
Exp. II		311	273	38	0	0
	Lesion	247	131	6	0	0
	counts/	549	134	135	1	0
	leaf	216	189	153	0	0
		249	240	173	1	0
		441	425	85	2	0
		595	114	221	1	0
		257	252	89	0	0
	Total	2865	1758	900	5	0
	Mean	358	220	113	1	0

TABLE 22. Lesion counts: Longevity of tobacco stunt agent infectivity in tobacco sap prepared in phosphate/PTC buffer and bioassayed on *Chenopodium amaranticolor*

		Incubation time (hours)						
		0	2	4	6	8	12	24
4°C		209	117	65	6	9	2	0
	Lesion	211	174	8	9	2	2	0
	counts/	271	167	17	7	2	0	0
	leaf	385	162	45	5	4	11	0
		169	218	108	47	17	2	0
		547	144	95	92	14	7	1
		484	91	173	70	12	26	2
		424	186	63	99	30	14	0
	Total	2700	1259	574	335	90	64	3
	Mean	338	157	72	42	11	8	0
20°C		387	1	0	0	0	0	0
	Lesion	178	0	0	0	0	0	0
	counts/	217	2	0	0	0	0	0
	leaf	372	5	0	0	0	0	0
		497	0	0	0	0	0	0
		471	0	0	0	0	0	0
		436	0	0	0	0	0	0
		326	0	0	0	0	0	0
	Total	2884	8	0	0	0	0	0
	Mean	361	1	0	0	0	0	0

TABLE 23. Lesion counts: Longevity of tobacco stunt agent infectivity in tobacco sap prepared in phosphate/PTC buffer plus 10 mg yeast RNA/ml and bioassayed on *Chenopodium amaranticolor*

		Incubation time (hours)									
		0	1	2	3	4	8	12	24	36	48
4°C		51	32	59	34	34	30	9	3	2	0
	Lesion	109	17	127	32	54	28	5	2	0	0
	counts/	99	37	149	31	32	6	14	1	0	0
	leaf	146	53	70	83	48	26	5	0	0	0
		203	130	34	32	22	11	18	4	0	0
		107	116	41	47	111	27	8	0	3	0
		118	115	34	112	61	17	12	1	0	0
		195	95	67	109	90	22	16	2	0	0
	Total	1028	575	581	480	452	167	87	13	5	0
	Mean	129	72	73	60	57	21	11	2	1	0
20°C		86	4	4	0	0	0	0	0	0	0
	Lesion	118	8	3	0	0	0	0	0	0	0
	counts/	98	0	0	0	0	0	0	0	0	0
	leaf	93	7	0	0	0	0	0	0	0	0
		136	23	1	0	0	0	0	0	0	0
		80	26	2	0	0	0	0	0	0	0
		193	22	3	0	0	0	0	0	0	0
		135	13	1	0	0	0	0	0	0	0
	Total	939	103	14	0	0	0	0	0	0	0
	Mean	117	13	2	0	0	0	0	0	0	0

TABLE 24. Lesion counts: Thermal inactivation point of tobacco stunt agent infectivity in tobacco sap bioassayed on *Chenopodium amaranticolor*

	Temperature (°C)													
	4°	20°	4°	25°	4°	30°	4°	35°	4°	40°	4°	45°	4°	50°
	69	75	77	44	99	41	149	0	96	0	137	0	196	0
Lesion	110	159	98	109	181	61	123	0	213	0	95	0	247	0
counts/	80	53	65	36	151	81	299	0	128	0	239	0	294	0
half leaf	48	49	49	47	35	9	306	0	185	0	169	0	259	0
	92	72	83	68	15	9	152	0	223	0	147	0	72	0
	53	31	114	166	232	52	88	0	145	0	201	0	178	0
	107	131	213	103	283	87	240	0	279	0	298	0	162	0
	184	246	167	99	138	8	349	0	136	0	256	0	206	0
Total	743	816	866	672	1134	348	1607	0	1405	0	1542	0	1614	0
Mean	93	102	108	84	142	44	201	0	176	0	193	0	202	0